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# COMPARISON OF THE GERMICIDAL EFFICIENCY OF HYPOCHLORITES OF HIGH AND LOW ALKALINITY

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The work of Mudge and Smith (1935) compared plate counts and serial dilution tubes in determining the efficiency of chlorine in destroying bacteria. They reported that, after exposure of the organisms to dilutions of a very highly alkaline hypochlorite, plate counts showed as high as a 99 per cent reduction while serial dilution tubes showed no reduction from the original bacterial population. They attribute their results to the fact that the chlorine dilutions exhibited bacteriostasis and not death. It is their belief that death of bacteria cannot be determined solely by the inability of the organisms to reproduce and cite the work of Rahn and Barnes (1933) to substantiate this theory. However, Fulmer and Buchanan (1923) using the vital staining technic, concluded that all cells which take up the stain are dead and therefore, can no longer reproduce, which has been the basic premise in studies of bacterial death.

Baylis (1931) believes that the action on cells by chlorine causes a permanent change, that there is no return to natural conditions, and that the reversible reaction as described by Bancroft and Richter (1931) probably does not apply to products of the chlorine group, or water supplies would not be as satisfactorily disinfected as results indicate.

Unpublished results of work done in our laboratory following both the Food and Drug Administration methods (Ruehle and Brewer, 1931) for testing antiseptics and disinfectants and the glass slide technique of Johns (1934) showed by cultural methods



as well as by animal inoculation (Costigan, 1936) that chlorine compounds destroy bacteria.

Butterfield (1933) found the plate colony counts and most probable numbers computed from the dilution method in agreement. Where there is a variation between the two methods (that is where the dilution method gives a higher count) he attributes it to the fact, that liquid media are probably more ideal for bacterial growth, and, more important, the organism under study may have a tendency to form clumps which on plate counts does not give a true representation of the numbers of bacteria present. Ziegler and Halvorson (1935) found the same phenomenon to be present in their work.

In view of the fact that alkalinity plays a very important part in the germicidal efficiency of hypochlorite solutions as was found by Johns (1931, 1934), Fabian and co-workers (1934), Mallmann (1932), Hellwig (1934), Levine and Charlton (1935), and Prucha (1934), and also because McCulloch (1936) found that highly alkaline products are less effective in destroying Gram-positive organisms than Gram-negative organisms, we thought it desirable to compare the efficiency of a highly alkaline hypochlorite<sup>1</sup> against one<sup>2</sup> of low alkalinity using the plate colony count and dilution tube methods. Attempts were made to use milk cultures (as did Mudge and Smith) but as we were unsuccessful in obtaining cultures without curdling, cultures of *Eberthella typhosa* (Hopkins strain) and *Staphylococcus aureus* (No. 209) were used.

The highly alkaline hypochlorite employed, had an available chlorine concentration of 15 per cent and an alkalinity, due to NaOH, of 0.5 per cent.

The hypochlorite of low alkalinity used, had an available chlorine concentration of 3.50 per cent with an alkalinity of 0.13 per cent due to  $\text{Ca}(\text{OH})_2$ .

The Leeds and Northrup glass electrode was used in determining the pH of the solutions.

<sup>1</sup> Chlor—Liquid Bleach—Manufactured by Pennsylvania Salt Manufacturing Company, Philadelphia, Pa.

<sup>2</sup> B-K—Manufactured by General Laboratories Division, Pennsylvania Salt Manufacturing Company, Philadelphia, Pa.

## TECHNIC

After three successive days of culturing in F.D.A. broth, (Ruehle and Brewer, 1931) the cultures were used in the test.

In order to simulate the work of Mudge and Smith (presence of milk in the inoculum), 1 cc. of sterile milk was added to 9 cc. of the 24-hour broth culture. 100-cc. portions of the two hypochlorites in dilutions containing 50, 100 and 200 parts of available

TABLE 1

*Comparison of the germicidal efficiency of a hypochlorite of high alkalinity against Eberthella typhosa by plate colony count and dilution methods*

TIME	HYPO-CHLORITE		COLONY COUNT NO THIOSULPHATE USED	PER-CENT-AGE REDUC-TION	COLONY COUNT THIOSULPHATE USED	PER-CENT-AGE REDUC-TION	GROWTH IN SERIAL TUBES	PER-CENT-AGE REDUC-TION
	Conc av. cl P.P.M.	pH of solution						
Start	50	9.09						
3 minutes	25	7.54	$7.8 \times 10^4$	99.99	$1.1 \times 10^4$	99.99	$1 \times 10^7$	99.90
5 minutes	20	7.54	$3.6 \times 10^4$	99.99	$4.9 \times 10^4$	99.99	$1 \times 10^6$	99.99
10 minutes	15	7.59	$6.0 \times 10^2$	99.99	$1.2 \times 10^3$	99.99	$1 \times 10^4$	99.99
Start	100	9.34						
3 minutes	65	7.84	2	99.99	3	99.99	$1 \times 10^3$	99.99
5 minutes	60	7.89	0	100	0	100	$1 \times 10^1$	99.99
10 minutes	55	7.89	0	100	0	100	0	100
Start	200	10.29						
3 minutes	135	8.29	0	100	0	100	$1 \times 10^2$	99.99
5 minutes	128	8.24	0	100	0	100	$1 \times 10^1$	100
10 minutes	120	8.24	0	100	0	100	0	100
3 minutes	Control	{	$2.9 \times 10^9$		$3.0 \times 10^9$		$1 \times 10^{10}$	
5 minutes			$2.9 \times 10^9$		$3.1 \times 10^9$		$1 \times 10^{10}$	
10 minutes			$2.9 \times 10^9$		$3.0 \times 10^9$		$1 \times 10^{10}$	

chlorine per million of water were used. The 10 cc. mixture of broth culture and milk was added to 100 cc. of hypochlorite dilution. At intervals of 3, 5 and 10 minutes, 1 cc. samples were withdrawn and run into sterile water and sterile N/10 sodium thiosulphate dilution bottles for plate counts on bacto-nutrient agar, and into each of 5 tubes of F.D.A. broth for serial dilution studies. The thiosulphate was used to stop the chlorine action at the stated intervals. Dilutions were made so that the plates were not overcrowded and satisfactory counts could be made.

Controls were run with each experiment using plate colony counts with both sterile water and sterile N/10 sodium thio-sulphate blanks as well as dilution tubes. The plates and tubes were incubated for 48 hours at 37°C.

The pH of the solutions and the available chlorine concentrations were determined at the beginning of the experiment and at 3, 5 and 10 minute intervals after the addition of the milk-culture inoculum.

TABLE 2

*Comparison of the germicidal efficiency of a hypochlorite of low alkalinity against Eberthella typhosa by plate colony count and dilution methods*

TIME	HYPO- CHLORITE		COLONY COUNT NO THIOSULPHATE USED	PER- CENT- AGE REDUC- TION	COLONY COUNT THIOSULPHATE USED	PER- CENT- AGE REDUC- TION	GROWTH IN SERIAL TUBES	PER- CENT- AGE REDUC- TION
	Conc av. cl P.P.M.	pH of solution						
Start	50	8.43						
3 minutes	35	6.94	$2.9 \times 10^5$	99.99	$6.2 \times 10^5$	99.97	$1 \times 10^6$	99.99
5 minutes	30	6.94	0	100	0	100	$1 \times 10^3$	99.99
10 minutes	30	6.94	0	100	0	100	$1 \times 10^2$	99.99
Start	100	8.53						
3 minutes	65	7.34	0	100	0	100	$1 \times 10^2$	99.99
5 minutes	60	7.39	0	100	0	100	$1 \times 10^1$	99.99
10 minutes	55	7.39	0	100	0		0	100
Start	200	8.85						
3 minutes	150	7.59	0	100	0	100	$1 \times 10^1$	99.99
5 minutes	140	7.69	0	100	0	100	$1 \times 10^1$	99.99
10 minutes	138	7.69	0	100	0	100	0	100
3 minutes	Control {		$2.7 \times 10^9$		$2.5 \times 10^9$		$1 \times 10^{11}$	
5 minutes			$2.7 \times 10^9$		$2.7 \times 10^9$		$1 \times 10^{10}$	
10 minutes			$2.7 \times 10^9$		$2.6 \times 10^9$		$1 \times 10^{11}$	

## DISCUSSION

As the results were very similar both for the control and hypochlorite treated tests, averages of six runs were considered satisfactory. Space does not permit showing each run of the experiment.

Table 1 shows the comparison of plate colony counts and dilution tubes using the highly alkaline hypochlorite against *Eber-*

*thella typhosa*. Of course, the colony count where thiosulphate was used, was higher than where water was used. There is a 99.9 per cent reduction in the counts as is shown by both plate counts and dilution tubes.

It will be noticed that the colony count even in the controls was consistently less than the count as estimated from the dilution tubes.

TABLE 3

*Comparison of the germicidal efficiency of a hypochlorite of high alkalinity against Staphylococcus aureus by plate colony count and dilution methods*

TIME	HYPOCHLORITE		COLONY COUNT NO THIOSULPHATE USED	PERCENTAGE REDUCTION	COLONY COUNT THIOSULPHATE USED	PERCENTAGE REDUCTION	GROWTH IN SERIAL TUBES	PERCENTAGE REDUCTION
	Conc. in ppm	pH of solution						
Start	50	9.09						
3 minutes	25	7.34	$5.0 \times 10^6$	99.83	$8.4 \times 10^6$	99.70	$1 \times 10^8$	99.0
5 minutes	20	7.34	$3.1 \times 10^6$	99.89	$4.2 \times 10^6$	99.84	$1 \times 10^7$	99.90
10 minutes	15	7.34	$6.6 \times 10^5$	99.97	$7.9 \times 10^5$	99.97	$1 \times 10^4$	99.99
Start	100	9.34						
3 minutes	63	7.64	$4.3 \times 10^6$	99.83	$6.0 \times 10^6$	99.75	$1 \times 10^8$	99.0
5 minutes	60	7.64	$8.4 \times 10^5$	99.97	$1.3 \times 10^6$	99.95	$1 \times 10^7$	99.90
10 minutes	58	7.69	$3.0 \times 10^4$	99.99	$6.5 \times 10^4$	99.99	$1 \times 10^5$	99.99
Start	200	10.29						
3 minutes	132	7.89	$1.2 \times 10^4$	99.99	$7.8 \times 10^4$	99.99	$1 \times 10^6$	99.99
5 minutes	125	7.90	$2.6 \times 10^3$	99.99	$3.0 \times 10^3$	99.99	$1 \times 10^5$	99.99
10 minutes	120	7.90	0	100	0	100	$1 \times 10^5$	99.99
3 minutes	Control	{	$2.9 \times 10^9$		$2.9 \times 10^9$		$1 \times 10^{10}$	
5 minutes			$3.0 \times 10^9$		$2.8 \times 10^9$		$1 \times 10^{11}$	
10 minutes			$2.9 \times 10^9$		$2.9 \times 10^9$		$1 \times 10^{11}$	

The hypochlorite of low alkalinity table 2, shows a greater efficiency against *Eberthella typhosa* by both methods.

In table 3, we find the comparison using *Staphylococcus aureus* with the highly alkaline hypochlorite. As is well known, the resistance of this organism is greater than that of *Eberthella typhosa*. Here again, we find the plate colony count and dilution tube methods to be in agreement. The plates where thiosulphate was used in the dilution blanks, show a higher count than where no thiosulphate was used.

Table 4 gives the results with the hypochlorite of low alkalinity against *Staphylococcus aureus*. We find a greater efficiency than with the highly alkaline hypochlorite. Again, it will be noticed that with the dilution method there is a 99.9 per cent reduction.

From the tables, it can be seen that the addition of the inoculum to both hypochlorites caused the greatest reduction in the available chlorine concentrations and pH values of the solutions

TABLE 4

*Comparison of the germicidal efficiency of a hypochlorite of low alkalinity against Staphylococcus aureus by plate colony count and dilution methods*

TIME	HYPO- CHLORITE		COLONY COUNT NO THIOSULPHATE USED	PER- CENT- AGE REDUC- TION	COLONY COUNT THIOSULPHATE USED	PER- CENT- AGE REDUC- TION	GROWTH IN SERIAL TUBES	PER- CENT- AGE REDUC- TION
	Conc av. cl P.P.M.	pH of solution						
Start	50	8.43						
3 minutes	30	6.80	$4.9 \times 10^6$	99.85	$8.1 \times 10^6$	99.73	$1 \times 10^8$	99.80
5 minutes	30	6.89	$1.2 \times 10^6$	99.96	$2.7 \times 10^6$	99.89	$1 \times 10^6$	99.99
10 minutes	30	6.89	$6.1 \times 10^4$	99.99	$9.5 \times 10^4$	99.99	$1 \times 10^5$	99.99
Start	100	8.53						
3 minutes	65	7.14	$3.4 \times 10^6$	99.87	$6.4 \times 10^6$	99.78	$1 \times 10^7$	99.99
5 minutes	55	7.14	$1.8 \times 10^5$	99.99	$3.3 \times 10^6$	99.98	$1 \times 10^5$	99.99
10 minutes	50	7.19	$1.6 \times 10^2$	99.99	$5.3 \times 10^2$	99.99	$1 \times 10^3$	99.99
Start	200	8.85						
3 minutes	145	7.34	0	100	0	100	$1 \times 10^3$	99.99
5 minutes	135	7.34	0	100	0	100	$1 \times 10^1$	99.99
10 minutes	135	7.34	0	100	0	100	$1 \times 10^1$	99.99
3 minutes	Control		$3.0 \times 10^9$		$2.0 \times 10^9$		$1 \times 10^{11}$	
5 minutes			$2.8 \times 10^9$		$2.9 \times 10^9$		$1 \times 10^{11}$	
10 minutes			$2.9 \times 10^9$		$2.9 \times 10^9$		$1 \times 10^{11}$	

in the first three minutes which then remained fairly constant through the ten-minute period.

#### CONCLUSIONS

The results show the plate colony counts and dilution methods to be in agreement and indicate that, with the hypochlorite dilutions used in the tests the organisms were definitely destroyed and therefore bacteriostasis was not a factor.

The dilution method gives a higher estimated bacterial population than the plate count method. This is probably due to the fact that a liquid medium is better adapted to bacterial growth; also, on agar plates, clumps of organisms may be present giving a lower colony count than would be estimated in dilution tubes.

A highly alkaline hypochlorite under the conditions of this test was found not to be as efficient germicidally as one of lower alkalinity.

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# THE FERMENTATION OF PROPYLENE GLYCOL BY MEMBERS OF THE ESCHERICHIA-AEROBACTER- INTERMEDIATE GROUPS

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The possibility has been suggested of differentiating the "intermediates" from *Escherichia* and *Aerobacter* by the use of propylene glycol (Dozois et al., 1936). It was observed, in the limited number of cultures studied, that *Escherichia* and *Aerobacter* formed acid from propylene glycol, while the "intermediates" did not. In order to determine whether the utilization of this glycol might serve as a convenient additional taxonomic characteristic of this group, extensive observations were deemed advisable. Therefore, the propylene-glycol broth medium was used, in the study of a number of stock cultures, and in the routine examination of strains isolated from water, milk, crabmeat, oysters and feces.

In an attempt to differentiate between the various strains of the "intermediates" observations on the fermentation of propylene glycol, ethylene glycol, adonitol and inositol were made. Those coli-form microorganisms which were found to be methyl-red positive and Voges-Proskauer negative, and which also grew in citrate broth (Koser's) were classed as members of the "intermediate" group.

## METHODS AND MATERIALS

One per cent of the specific sugar alcohol was added to sugar-free broth of a pH of 7.0. The indicator used was chlorphenol



red. The broth was sterilized at 15 pounds for 15 minutes, excessive heating being avoided. The cultures were incubated for ten days and observed daily for acid and gas. In most instances 48 hours' incubation was sufficient for fermentation to take place.

## RESULTS

Of the 33 cultures of the "intermediates" obtained from various stock collections, 16 failed to form acid from either propylene glycol or ethylene glycol, and 4 failed to form acid from propylene glycol but did produce acid from ethylene glycol. Of the 16 cultures giving negative results with both of the glycols, 5 produced acid and gas from adonitol and inositol. Of the 13 strains that formed acid from propylene glycol, 7 also produced acid from ethylene glycol, while all failed to affect adonitol and inositol (table 1, A).

Forty-five cultures of "intermediates" were isolated from oysters, crabmeat<sup>1</sup> and feces (see table 1, B). Of these, 21 were found to be both propylene-glycol and ethylene-glycol negative. Three that were negative with propylene glycol produced acid from ethylene glycol. Five of the group of 21 organisms fermented adonitol and inositol while all of the propylene-glycol negative, ethylene-glycol positive strains attacked adonitol and inositol. The other members of this group produced acid from propylene glycol; of these, 11 also formed acid from ethylene glycol. Only 1 of the organisms positive in both of the glycol broths fermented adonitol and inositol while 3 of the propylene-glycol positive and ethylene-glycol negative strains have this ability.

Of the "intermediates" isolated from milk and water at the Baltimore City Health Department (table 1, C) 9 failed to form acid from propylene and ethylene glycol. Thirty-three produced acid from propylene glycol, of which 21 also formed acid from ethylene glycol.

Approximately 44 per cent of the "intermediates" studied failed to form acid from propylene glycol. Of those that failed to utilize the propylene glycol about 13 per cent formed acid

<sup>1</sup> T. F. Dozois, Maryland State Department of Health.

from ethylene glycol. Thirty-two and a half per cent of the "intermediates" produced acid from propylene and ethylene glycol while 23.3 per cent produced it only from propylene glycol. About 11 per cent of the "intermediates" fermented adonitol and inositol. All of those that fermented adonitol also fermented inositol.

TABLE 1

*Observations on the reactions of Escherichia coli and the "intermediates"*

TOTAL NUMBER OF CULTURES	PROPYLENE GLYCOL ACID	ETHYLENE GLYCOL ACID	CITRATE GROWTH	INDOL POSITIVE	ADONITOL ACID AND GAS	INOSITOL ACID AND GAS
A. "Intermediates" from various stock collections						
7	7	7	7	7	—	—
6	6	—	6	6	—	—
16	—	—	16	4	5	5
4	—	4	4	4	1	1
B. "Intermediates" from oysters, crabmeat and feces						
11	11	11	11	11	1	1
10	10	—	10	10	3	3
21	—	—	21	11	5	5
3	—	3	3	—	3	3
C. "Intermediates" from milk and water						
21	21	21	21	8	—	—
12	12	—	12	12	4	4
9	—	—	9	9	—	—
D. <i>Escherichia coli</i>						
Fecal 17	—	—	—	17	—	—
Non fecal 17	1	—	—	17	—	—

It is apparent from these observations that there are at least four distinct strains of the "intermediates" in so far as the glycol fermentations would indicate. It will be necessary for more detailed studies to make this classification complete.

Thirty-four strains of *Escherichia coli* (table 1, D), 17 from feces and 17 from other sources, were obtained. Only one produced

acid from propylene glycol. From the studies on these cultures of *Escherichia coli* it would seem that a close correlation exists between the failure to utilize citrate and the failure to produce acid from propylene glycol. While the indol reaction for members of the *Escherichia* and "intermediates" group is somewhat variable it would seem that a relationship between indol formation and propylene-glycol utilization by *Escherichia coli* may exist. The production of indol in most cases signifies an inability to ferment the glycol.

#### DISCUSSION

Over half of the "intermediates" from various stock collections and slightly less than half of those isolated from oysters, crab-meat and feces failed to utilize propylene glycol. Of the 42 strains isolated from milk and water 33 produced acid from propylene glycol. These variations and the differences observed from the *Escherichia coli* suggest that the ability to utilize propylene glycol is a strain characteristic. The source of isolation may play a part in the propylene-glycol activity of the organisms.

Although several *Escherichia coli* in our stock collection produce acid from propylene glycol, of the 34 isolated from fresh material only one has this ability. More extensive studies of the action of *Escherichia coli* from a large number of sources will be necessary.

The colon-typhoid group of organisms which yield lactic or formic acid from glucose might be expected to yield lactic, formic or propionic acids or possibly mucic, oxalic or acetic acids from propylene glycol. An attempt was made to identify the acid formed by these organisms from propylene glycol. The total acidity was approximately one half that yielded by glucose. One cubic centimeter of broth medium required 0.1 cc. N/10 NaOH for neutralization. Negative reactions were obtained for lactic, oxalic and mucic acids. No volatile acids were present, thus eliminating acetic or formic acids.

#### SUMMARY

1. Because of the variation of reactions of *Escherichia coli* and the "intermediates" with propylene glycol, this method, in its present form, can not be used for differentiation.

2. A close correlation exists between the failure to utilize citrate and to ferment propylene glycol by *Escherichia coli*.

3. A relationship between indol formation and propylene-glycol fermentation by *Escherichia coli* has been observed.

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# THE DISSIMILATION OF ORGANIC ACIDS BY AEROBACTER INDOLOGENES<sup>1</sup>

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In a previous paper by Reynolds and Werkman (1937) evidence was presented indicating that acetic acid plays an important intermediary rôle in the dissimilation of glucose by *Aerobacter indologenes*. It was suggested that the products of the anaerobic dissimilation of intermediately-formed acetic acid are acetyl-methylcarbinol and 2,3-butylene glycol. In view of the indirect evidence previously reported and the possible importance of the behavior of acetic acid in the Voges-Proskauer reaction, it appeared desirable to provide direct proof of that suggestion.

## METHODS

Fermentations were carried out in 1-liter flasks filled with medium and fitted with delivery tubes for removal of gases. Carbon dioxide was collected in "U" tubes filled with soda-lime, and weighed. Hydrogen was collected over water in graduate cylinders and quantitatively determined in a Hempel explosion pipette.

Other methods are described in a previous paper (1937).

## EXPERIMENTAL

Investigation of the dissimilative action of *A. indologenes* on acetic, lactic and succinic acids was undertaken. Preliminary attempts to ferment these acids in the form of their sodium salts in a peptone medium, were unsuccessful. Clouding of the medium and microscopic examination indicated bacterial growth but

<sup>1</sup> Supported in part by a grant from the Industrial Science Research Fund of Iowa State College.

chemical analysis demonstrated a negligible utilization of the acids. None of the media gave a positive acetylmethylcarbinol reaction. In succeeding experiments, dissimilation was obtained in media containing glucose in addition to the organic acids. In the presence of glucose conditions are more nearly those of a normal fermentation, particularly as regards possible hydrogen transfer. To the medium containing 2.0 per cent glucose, 0.1 per cent secondary ammonium phosphate, and 0.5 per cent sodium bicarbonate, was added 0.5 per cent of the acid as its sodium salt. A quantity of the same medium without addition

TABLE 1

*Fermentation of glucose plus an organic acid by Aerobacter indologenes\**

ADDITION	INITIAL ACID	CO <sub>2</sub>	H <sub>2</sub>	FORMIC ACID	ETHYL ALCOHOL	ACETIC ACID	LACTIC ACID	ACETYL METHYL CARBINOL	2,3-BUTYLENE GL'COL	SUCCINIC ACID	CARBON RECOVERY	OXIDATION-REDUCTION INDEX†
											per cent	
None..	0	1 53	0 28	0 28	0 67	0 01	0 03	0 00	0 64	0	97	0 943
Acetic acid..	0 86	1 71	0	0 09	0 55	0 50	0 03	0 02	0 88	0	98 6	0 953
Lactic acid..	0 57	1 64	0 39	0 27	0 61	0 11	0 62	0 01	0 68	0	103 5	0 970
Succinic acid..	0 43	1 49	0 31	0 38	0 65	0 04	0 02	0 00	0 72	0 28	95	0 875

\* Quantities expressed as moles per mole of glucose fermented

† Cf. Erb, Wood and Werkman: Jour. Bact., **31**, 595 (1936); a perfect index = 1.0.

of acid served as the control. Following inoculation, sterile, oxygen-free nitrogen was forced for fifteen minutes through the flasks, which were connected immediately to the gas train. Samples were removed for determination of the initial glucose, carbon dioxide and acid. Final analyses were made after seven days' incubation at 30°C. Typical results are shown in table 1. Fermentation of glucose was complete. Examination of the carbon balance and oxidation-reduction index indicates that the analyses were satisfactory.

The data show that added lactic acid was not attacked. The

increase in lactic acid over the original addition is comparable with the quantity produced by the control fermentation of glucose with no additions.

Comparison of the initial and final quantities of acetic acid in the glucose-acetate medium shows that 0.36 mole of the added acid has disappeared. Hence, a quantity of acetic acid equivalent to 40 per cent of the original addition plus that produced from the glucose was converted to some other product or products. The greatest variations from the control accompanying the utilization of acetic acid are in formic acid, acetylmethylcarbinol, 2,3-butylene glycol and hydrogen. Since the total quantities of 1-, and 3-carbon compounds are practically equal in the control and in the glucose-acetate medium, and the quantity of ethyl alcohol is less in the latter, the disappearance of acetic acid can be explained only on the basis of its conversion to 4-carbon compounds, i.e., 2,3-butylene glycol and acetylmethylcarbinol.

The increase in 2,3-butylene glycol in the acetate medium as compared with the control is 0.24 mole. The decrease in acetic acid calculated as 2,3-butylene glycol is 0.18 mole or equal to 75 per cent of the increase in yield of glycol.

The data show a significantly decreased production of alcohol in the presence of acetate. Since it is probable that the alcohol arises through the reduction of intermediately-formed acetaldehyde, such a decreased yield should be expected in the presence of a competing hydrogen acceptor, i.e. acetic acid. As with the converted acetic acid, intermediately-formed aldehyde not reduced to alcohol must be accounted for among the 4-carbon compounds. The decrease in ethyl alcohol in the glucose-acetate medium as compared with the control is the equivalent of 0.055 mole of 2,3-butylene glycol and equal to 23 per cent of the increase in yield of glycol. Thus, 98 per cent of the increased yield of glycol in the presence of acetate can be accounted for by the converted acetic acid plus the condensation of intermediate aldehyde normally reduced to alcohol.

That acetic acid can be activated as a hydrogen acceptor by *A. indologenes* is shown by the absence of gaseous hydrogen in the presence of an excess of that acid. The utilization of hydro-



gen arising from the dissimilation of glucose by *A. indologenes* was substantiated by use of the Barcroft-Warburg respirometer. The technique is that described by Dixon (1935) with oxygen-free nitrogen substituted in the flasks for air. The results showed that in the presence of acetate no appreciable quantity of hydrogen may be liberated by *A. indologenes* whereas *Escherichia coli*, used for comparison, behaves otherwise. This is a fundamental difference between the two organisms.

Results obtained with the fermentation of added succinic acid are not as definite as those for acetic acid. About 40 per cent of the initial succinic acid was fermented and its conversion was

TABLE 2

*Fermentation of a glucose-acetate medium by Aerobacter indologenes in the presence of hydrogen, and hydrogen and air*

TREATMENT	INITIAL ACETIC ACID	FINAL ACETIC ACID	CARBON DIOXIDE	FORMIC ACID	ACETYL METHYL CARBINOL	ETHYL ALCOHOL	2, 3-BUTYLENE GLYCOL	CARBON RECOVERY	OXIDATION INDEX REDUCTION
							per cent		
Hydrogen	1.63	1.36	1.72	0.28	0.010	0.53	0.86	100.1	1.02
Hydrogen and air	1.63	1.47	1.73	0.14	0.01	0.44	0.82	97.4	1.08

Quantities in moles per mole of glucose fermented.

accompanied by appreciable increases in formic acid and 2,3-butylene glycol. The results indicate that the fermented succinic acid was converted largely to formic acid and 2,3-butylene glycol. Conversion to the glycol probably occurs subsequent to an initial decomposition to acetaldehyde or acetic acid.

Since less than half of the acetic acid initially present was hydrogenated, the reduction appears to have been limited by lack of available hydrogen. The data suggested the possibility of obtaining more complete reduction by providing an excess of hydrogen. To test that possibility the following experiment was arranged. One liter of medium containing 1.0 per cent of acetic acid as the sodium salt with 0.5 per cent Bacto-peptone was

inoculated with *A. indologenes*. Hydrogen was forced through the medium during six days' incubation at 30°C. Two other flasks containing the glucose-inorganic medium as previously described and 1.0 per cent of acetic acid as the sodium salt were inoculated with the same culture. Hydrogen was forced through one of these, and a mixture of equal parts of hydrogen and oxygen through the other during the six days' incubation at 30°C. The flasks were fitted with inlet tubes extending to the bottom. The end of the inlet tube was inserted tightly into a small basswood block. The gases, forced through the block, entered the medium as a fine spray.

The peptone-acetate medium showed good growth but the added acetic acid was entirely recovered unchanged after incubation. In the glucose-acetate medium (table 2), the fermentation of glucose was complete.

Comparison of the data in tables 1 and 2 shows that substantially equivalent quantities of acetic acid were reduced in the absence and in the presence of gaseous hydrogen. The results indicate that under the conditions molecular hydrogen was not activated to serve in the reduction of acetic acid. Apparently a more active form is required. In substantiation we have found by means of the Thunberg technique that molecular hydrogen is not activated by *A. indologenes* with methylene blue as acceptor. In the presence of a mixture of oxygen and hydrogen less acetic acid was reduced and reduction products (ethyl alcohol and 2,3-butylene glycol) were somewhat lower. Although hydrogen could not be determined on these fermentations the oxidation-reduction indexes, being close to 1.0, indicate that no appreciable quantities of hydrogen were liberated. The data in table 2 show that acetic acid is more readily activated as a hydrogen acceptor by *A. indologenes* than is molecular oxygen.

#### SUMMARY

*Aerobacter indologenes* dissimilates acetic or succinic but not lactic acid in the presence of glucose and under anaerobic conditions.

Although gaseous hydrogen is a product of the fermentation of

glucose by *A. indologenes*, no appreciable quantities are produced when glucose is fermented in the presence of sufficient acetate. *Escherichia coli* behaves differently.

The evidence indicates that in the case of *A. indologenes* acetic acid is reduced and condensed to 2,3-butylene glycol, probably through acetaldehyde and acetylmethylcarbinol as intermediary stages. Molecular hydrogen is not activated to reduce the acid.

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# STUDIES ON HEMOLYTIC STREPTOCOCCI

## IV. STREPTOCOCCUS SCARLATINAE

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### INTRODUCTION

In the second paper of this series *Streptococcus pyogenes* was described, and it was stated that among the 120 scarlet fever strains in our collection, 74, or 61.6 per cent, had the characters of that species. Strains from a great variety of other streptococcus diseases also agreed with *S. pyogenes*. It was stated that another group included only scarlet fever strains. That group will be described in this paper.

### NOMENCLATURE

On account of the technical limitations of the early bacteriologists, they were unable to describe strains fully enough to be identified with groups recognized as species many years later. It is logical, however, to apply the name *Streptococcus scarlatinae*, which Klein gave in 1887 to his scarlet fever strains, to a group which appears to be specific for scarlet fever. (In the earlier paper the reasons were given for not applying the name *S. scarlatinae* to the *S. pyogenes* group which, according to our data, appears to be the most common cause of scarlet fever.)

Andrewes and Horder were the first to recognize a distinctive character of a group of streptococci which they observed to be associated particularly with scarlet fever. They described the type as capable of fermenting sucrose, lactose, and raffinose, but not inulin, salicin, coniferin, or mannitol. They considered the

likelihood that this group of organisms was the same as Klein's *S. scarlatinae*, but rejected the idea on the basis of the frequent incapacity of their streptococcus for growth on gelatin at 20°C. They gave it the name *Streptococcus anginosus*. Writing before it had been established that streptococci can produce scarlet fever, Andrewes and Horder stated that it seemed likely that if scarlet fever is a streptococcal disease some form of *S. anginosus* would be found to be the causal agent.

Andrewes and Horder did not offer a clear definition of *S. anginosus*, for, as the data presented in this paper will show, differences in ability to grow at 20°C. are not sufficiently definite to be regarded as a differential test for the classification of scarlet fever strains. Hence, the introduction of the new name *S. anginosus* for the salicin-nonfermenting group was not justifiable. Since it has been commonly used, however, it must now be regarded as a synonym of *S. scarlatinae*.

Andrewes and Horder described 25 variants of *S. anginosus* which differed from their type strain in fermentation reactions. Ten of the variants were capable of fermenting salicin. They found their poorly-defined *S. anginosus* not only in scarlet fever, but occasionally also in other sore throats, as well as in other diseased conditions. They observed, however, that it is rarely associated with suppuration. According to our data, it appears that Andrewes and Horder would not have found such a wide distribution of *S. anginosus* in other than scarlet fever and sore throat cases if they had limited the species to strains incapable of fermenting salicin.

Holman defined the species *S. anginosus* more clearly by limiting it to strains capable of fermenting lactose but incapable of fermenting mannitol or salicin. He confirmed the observation of Andrewes and Horder that few strains of this group are found to be the causative agents in purulent conditions.

The preceding review has shown how the specific name *anginosus* came into use for the group of streptococci to which, as it now appears, the name *S. scarlatinae* should apply. For the following reasons the writer accepts the specific name *S. scar-*

*latinae*<sup>1</sup> for the group of streptococci associated with scarlet fever and distinguished by inability to ferment salicin:

(1) It was the first specific name given to streptococci associated with scarlet fever; (2) it is a suitable name, implying the specificity for scarlet fever which, as will be shown later, the group possesses; (3) a strain ("N. Y.5") of this group, designated as *S. scarlatinae* in the Catalog of the American Type Culture Collection, is commonly used by the manufacturers for the production of scarlet fever antitoxin, on account of the potency and polyvalency of its toxin.

A number of writers have designated as *S. scarlatinae* any streptococcus which fails to ferment salicin. The errors resulting from relying on fermentation reactions alone for the classification of streptococci were discussed in the preceding paper of this series. It happens, however, that according to our data, although there are other groups of hemolytic streptococci associated with other diseases which fail to ferment salicin, nevertheless among scarlet fever strains failure to ferment salicin is a distinctive character. In our collection of 395 strains of hemolytic streptococci from human disease sources, the strains belonging to the species *S. scarlatinae* are the only ones which fail to ferment salicin, excepting the strains of one small group which disagrees with *S. scarlatinae* in other characters and appears to be incapable of causing scarlet fever, and some of the strains of "minute hemolytic streptococci" of Long and Bliss which, on account of their very slow and sparse growth, would hardly be confused with *S. scarlatinae*. Hence, whenever salicin-nonfermenting scarlet fever streptococci are mentioned in the literature, it is reasonably certain that *S. scarlatinae* is the species under discussion.

<sup>1</sup> There is a question in the mind of the writer as to whether it might not be more logical to regard the group of salicin-nonfermenting strains as a variety or sub-species of *S. pyogenes*. Since, however, bacteriologists generally regard every clearly defined group as a separate species, and since in writing or speaking it is awkward to give species and sub-species designation, the group is referred to as a species in this paper. At any rate the name *scarlatinae* is the valid name for the salicin-nonfermenting group of scarlet fever streptococci, whether the group be regarded as a species or a variety.

THE CHARACTERS OF *STREPTOCOCCUS SCARLATINAE*

The differential characters<sup>2</sup> of *S. scarlatinae* are like those of *S. pyogenes* (see the second paper of this series), excepting that *S. scarlatinae* does not ferment salicin. The characters of the type strains of the two species are given in table 1. Although the two species are distinguished by only one of the differential tests, this test gives an obviously valid distinction, because inability to ferment salicin is correlated with definite pathogenic properties. Our data show that *S. pyogenes* is capable of causing

TABLE 1

The differential characters of the type strains of *S. pyogenes* and *S. scarlatinae*

	DIFFERENTIAL CHARACTERS										
	Designation of type strain	Sensitivity to nascent phage				Sensitivity to B 563 filtrate	Fermentation of				
		B 563	C 594	C 646	D 693		Lactose	Salicin	Mannitol	Trehalose	Sorbitol
<i>S. pyogenes</i>	1168	+	+	+	—	—	+	+	—	+	—
<i>S. scarlatinae</i>	612	+	+	+	—	—	+	—	—	+	—

	OTHER CHARACTERS					
	Fatal pH in glucose broth	Growth in bile		Hydrolysis of sodium hippurate	Lysis of human fibrin	Virulence for mice
		10 per cent	40 per cent			
<i>S. pyogenes</i> . . . . .	5.4	—	—	—	++++	10 <sup>-4</sup>
<i>S. scarlatinae</i> . . . . .	5.4	+	—	—	+	10 <sup>-3</sup>

not only scarlet fever, but also erysipelas, puerperal fever, and many kinds of acute suppurative diseases, whereas it appears that *S. scarlatinae* may be specific for scarlet fever, (or sore throat without the rash,) for in our collection of 262 strains of hemolytic streptococci from all kinds of human streptococcus diseases other than scarlet fever, not one agreed with *S. scarlatinae*.

In addition to the one differential test which distinguishes

<sup>2</sup> The methods for determining the characters were described in the first paper of this series.

*S. scarlatinae* from *S. pyogenes*, there are other differences which may be observed when a number of strains of the two species are compared, but they are not sufficiently definite to be useful for the identification of individual strains.

On 10-per-cent-bile blood agar 42.8 per cent of 14 strains of the *S. scarlatinae* group grew as compared with 17.6 per cent of the 74 scarlet fever strains of the *S. pyogenes* group.

Nineteen scarlet fever strains of *S. pyogenes* and thirteen strains of *S. scarlatinae* were compared as to their ability to dissolve human plasma. The results summarized in table 2 show that, in general, the strains of *S. scarlatinae* possess weaker fibrinolytic power than the scarlet fever strains of *S. pyogenes*.

TABLE 2

*Comparison of fibrinolytic properties of scarlet fever strains of the species S. pyogenes and S. scarlatinae*

DEGREE OF FIBRINOLYSIS	S. PYOGENES (19 STRAINS TESTED)		S. SCARLATINAE (13 STRAINS TESTED)	
	number	per cent	number	per cent
++++	4	21.1	0	0
+++	1	5.2	0	0
++	8	42.1	2	15.4
+	5	26.3	7	53.8
—	1	5.3	4	30.8

The virulence of *S. scarlatinae* for mice is low. Among 9 strains which had been maintained in laboratories for years, 7 failed to kill mice in the lowest dilution tested (1:100); 2, including the type strain, killed mice in the 1:1000 dilution of the passage strain.

The observation of Andrewes and Horder that salicin-nonfermenting strains would not grow at 20°C. could not be confirmed. Of twelve strains of *S. scarlatinae* examined for this character, all grew at 20°C., as did also a similar number of strains of *S. pyogenes*. No difference could be observed in the rate of growth of the strains of the two species at this temperature.

The serological classification of representative strains of *S. scarlatinae* has been determined by several investigators. Two



strains of our collection (nos. 642 and 646) were studied serologically by Williams, and also by Griffith. Williams placed them in her agglutinative type 3, and stated that all of her scarlet fever strains of type 3 belonged to the *anginosus* group, failing to ferment salicin. Griffith placed strains 642 and 646 in his type 10, which appears to be the same as Williams' type 3. It would appear from the work of these two investigators that the strains of the species *S. scarlatinae* all belong to the same agglutinative type, whereas the strains of *S. pyogenes* belong to a number of agglutinative types, as was shown in the previous paper.

Dr. Elizabeth Verder, associated with the writer, has studied 10 *S. scarlatinae* strains and found that they all belong to Lancefield's group A.

#### THE TYPE STRAIN

It seemed logical to choose for the type strain of *S. scarlatinae* the one which, on account of its polyvalency and the high potency of its toxin, has been widely distributed among laboratories for the production of scarlet fever antitoxin. Hence, strain 642, isolated many years ago from a case of scarlet fever by Dochez and known in the literature as "N.Y.5," was chosen for the type strain. It has been deposited in the American Type Culture Collection where it was already listed as *S. scarlatinae* no. 4543.

Strain 642 is sensitive to phages B/563, C/594 and C/646 in the nascent state; it is not sensitive to D/693 in the nascent state, or to lytic filtrate B/563; under the conditions of the test, final pH in glucose broth is 5.4; lactose and trehalose are fermented; salicin, mannitol, and sorbitol are not fermented. Growth occurs on 10-per-cent- but not on 40-per-cent-bile blood agar; sodium hippurate is not hydrolyzed; fibrinolysis of human plasma is weak, occurring between the 3rd and 24th hours or failing; mice are killed in the  $10^{-3}$  dilution in a series of rapid passages.

The cells of strain 642 are Gram-positive, and in broth culture they occur in pairs or chains, many of which are long. On infusion agar containing 5-per-cent rabbit blood, after 48 hours' incubation, the colonies of the stock strain are smooth, convex,

discrete. The largest isolated colonies are about 1 mm. in diameter. A zone of clear hemolysis about 1 mm. wide surrounds the colonies.

#### THE SOURCES OF *S. SCARLATINAE* STRAINS

Our collection contains 14 *S. scarlatinae* strains. They were from the following geographical sources:

From the United States, 5 strains; from Argentina, 3 strains; from Austria, 3 strains; from Hungary, 2 strains; from Russia, 1 strain.

The previous designations of our strains belonging to the species *S. scarlatinae* which have been studied by other investigators are as follows: Dochez' N.Y. 5 and 53 (American Type Culture Collection nos. 4543 and 4014); Williams' no. 57, type III; Dick IV; Michigan Department of Health no. 322; Andrewes and Christie's "Moscow 81." At least four of these strains are used by various laboratories for the production of antitoxin.

The four strains designated as *S. scarlatinae* listed in the Catalog of the American Type Culture Collection (third edition, 1934) were studied. As stated above, two were found to have the characters of *S. scarlatinae*. The other two were found to belong to other streptococcal species.

#### PREVALENCE OF *S. SCARLATINAE*

Fourteen, or 10.5 per cent of our 133 scarlet fever strains agreed with *S. scarlatinae*. Williams reported about the same percentage of salicin-nonfermenting strains in her collection. Seven out of 68, or 10.7 per cent of her scarlet fever strains failed to ferment salicin and agreed with her agglutinative type 3. Bliss found no strains which failed to ferment salicin among the 25 scarlet fever strains which he studied.

Apparently *S. scarlatinae* was not common in England when Griffith collected his strains of streptococci. He found no scarlet fever strains belonging to his type 10, which, as already pointed out, appears to be made up of *S. scarlatinae* strains. However, he isolated strains of his type 10 from 12 cases in a school outbreak of sore throat.

Although Griffith failed to find *S. scarlatinae* among the English

scarlet fever strains, it seems to have been prevalent in England three decades earlier, when Andrewes and Horder were so impressed with the frequency of salicin-nonfermenting strains in scarlet fever throats that they predicted that if it would ever be shown that a streptococcus is the etiologic agent in scarlet fever, *S. anginosus*, as they called it, would be found to be the one concerned. Thus, it appears, there was a wave of *S. scarlatinae* infection spread over England in 1906, but this organism was uncommon in England when Griffith collected the streptococci which he studied. In later papers of this series similar waves of other species of hemolytic streptococci will be noted.

Hence, although the geographical sources of our strains show that *S. scarlatinae* is widely distributed, our data and the information in the literature lead to the conclusion that *S. scarlatinae* is not always and everywhere a common cause of scarlet fever.

#### THE DISEASE SPECIFICITY OF *S. SCARLATINAE*

As previously stated, Andrewes and Horder observed a relationship between the presence of *S. anginosus* in the throat and symptoms of scarlet fever. They also noted that *S. anginosus* was only rarely associated with suppuration. They found it in disease conditions other than scarlet fever, but as we have already pointed out, the wider distribution they reported, as compared with the marked disease specificity which we found, can be explained by the fact that Andrewes and Horder considered certain salicin-fermenting strains to be variants and reported them as *S. anginosus*.

It is more difficult to understand why Holman found *S. anginosus* in a variety of disease conditions, for he limited the species to salicin-nonfermenting strains. However, Holman confirmed the observation of Andrewes and Horder that remarkably few strains of this group could be definitely classed as the causative agent in purulent conditions, but that they are frequently associated with scarlet fever.

According to our data, it would appear that *S. scarlatinae* has a disease specificity, for among 262 strains of hemolytic streptococci from human diseases other than scarlet fever, not one

agreed with *S. scarlatinae*. From the observation of Griffith, already quoted, however, and from the following observation of Williams, there seems to be no doubt that *S. scarlatinae* may cause sore throat without a rash. Williams reported that 4 strains of streptococci from cases of septic sore throat during an epidemic were found to agree with *S. anginosus* and with her type 3 agglutinative group. The epidemic occurred among the members of the staff of a contagious disease hospital, and was thought to have started from a case of scarlet fever. No rash developed in the sore throat subjects, all of whom had had scarlet fever, or had been recently immunized with scarlatinal vaccine.

#### CAN *S. SCARLATINAE* INFECT COWS?

None of the strains of *S. scarlatinae* in our collection was from a milk-borne epidemic. The present study is not extensive enough, however, to conclude from these data that *S. scarlatinae* may not infect the cow's udder. The literature yields no information on this point. Frost, Gumm and Thomas described a milk streptococcus characterized by inability to ferment salicin, which differed from *S. scarlatinae*, however, in its ability to produce a higher degree of acidity in broth culture, and in its ability to hydrolyze sodium hippurate. These characters indicate that their strains were of the animal type.

Minett and Stableforth also described salicin-nonfermenting strains of hemolytic streptococci from cow's milk. They were peculiar strains, capable of fermenting both trehalose and sorbitol. In their ability to produce acid in sorbitol they differed from *S. scarlatinae*. Smith and Brown and also Jones reported that they found in cow's milk streptococci incapable of fermenting salicin. Their studies were made before the usefulness of trehalose and sorbitol for the differentiation of human and animal strains had been discovered. It now appears probable that their strains were of the animal type.

#### DISCUSSION

Having classified 61.6 per cent of scarlet fever strains of our collection in the species *S. pyogenes* (see first paper of this series),

and 10.5 per cent in the species *S. scarlatinae*, there remain a considerable percentage of scarlet fever strains which do not agree with either of these two species. They belong to other species which will be described in later papers of this series.

The confusion which resulted from basing the classification of hemolytic streptococci on disease source should be dispelled when disease problems are reconsidered in the light of the newer knowledge of streptococcal classification. The fact that different species of streptococci may be involved in different scarlet fever epidemics offers a possible explanation as to why the character and severity of the disease, the occurrence of secondary infections, and the mortality rates are subject to wide variations in different epidemics.

Considering the observations of the pyogenic property of *S. pyogenes* and the lack of this property in *S. scarlatinae*, it may be expected that complications will be found to occur more commonly in scarlet fever epidemics caused by *S. pyogenes* than in those caused by *S. scarlatinae*. An observation made in the Contagious Disease Hospital, Chicago, by Dack, Woolpert and Hoyne is of interest in this connection. They found that the strains from complicated cases of scarlet fever are more actively fibrinolytic than strains from uncomplicated cases. Their observation is harmonious with our observation that the strains of *S. pyogenes* are more actively fibrinolytic than the strains of *S. scarlatinae*.

So many difficulties attend the classification of hemolytic streptococci according to toxin production that a full understanding of the quality of toxin produced by the several species of scarlet fever strains is impossible at this time. The quality of toxin produced by streptococci is a definite character, however, and a practical method for qualitative determinations on large numbers of cultures will probably be found sometime. The limited data at hand suggest that grouping according to toxin production is correlated with other characters, as was to be expected. If, in the future, any group which is described as a species in the present series of papers is found to include more than one group as deter-

mined by toxin production, the group would then be further subdivided accordingly.

Although the species *S. scarlatinae* includes only 10.5 per cent of scarlet fever strains, Wadsworth and Coffey found that the antiserum produced by the type strain neutralized the toxin produced by 67.3 per cent of scarlet fever strains. Hence, if the strains of the species *S. pyogenes*, which when judged by our collection includes 61.6 per cent of scarlet fever strains, all produce toxin of the same neutralizing quality, the corollary statement may be made, that the toxin produced by strains of the species *S. pyogenes* must be neutralized by antitoxin produced by "N.Y.5." As a matter of fact, the sum of the percentages of the scarlet fever strains of the two species in our collection ( $61.6 + 10.5 = 72.1$ ) gives a figure as close to the percentage of scarlet fever strains found by Wadsworth and Coffey to be neutralized by "N.Y.5" serum as would be expected when two collections are compared with reference to a single character.

There are in our collection of *S. pyogenes* 23 strains, the toxin of which has been studied by other investigators and found to be neutralized by "N.Y.5" antitoxin. Three of the strains were from cases of scarlet fever; ten from erysipelas; two from puerperal fever; two from septic sore throat; two from cases of rheumatism; one from suppurative arthritis; one from pleurisy; and two were from cow's milk isolated during septic sore throat epidemics. The toxin of three of the 23 strains was studied by Eagles; that of two strains was studied by Williams and Gurley; that of two strains was studied by Wadsworth and Coffey. M. V. Veldee of this laboratory studied 16 of the 23 *S. pyogenes* strains, carrying out the neutralization test on the rabbit's ear. The writer is indebted to him for permitting this statement to be made from unpublished data. Dr. Veldee also examined two other *S. scarlatinae* strains (Dick IV and Williams type 3, no. 57) and found that their toxin is neutralized by "N.Y.5" antitoxin.

#### SUMMARY

In our collection of 395 strains of hemolytic streptococci from various human diseases, there is a group of 14 strains, all

from cases of scarlet fever, which is distinguished from *Streptococcus pyogenes* by lack of ability to ferment salicin. The group is described in this paper. From a review of the literature it is concluded that the logical designation for this group is *Streptococcus scarlatinae*. The species name "*anginosus*" is a synonym.

Agreeing with the findings of other investigators, the data show that *Streptococcus scarlatinae* may cause scarlet fever or sore throat without rash, but that it is rarely, if ever, the cause of other diseases. The strains of this group are weak in their ability to dissolve human fibrin.

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# APPEARANCE OF DOUBLE-ZONE BETA HEMOLYTIC STREPTOCOCCI IN BLOOD AGAR

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In 1913 Smith and Brown (1915) isolated from the milk of four cows in a herd where mastitis was prevalent, four strains of streptococci with the hemolytic properties to be described. In the primary blood agar plate the deep colony of one strain was surrounded by "a clear inner circle (of hemolysis) three millimeters in diameter and a partly cleared outer zone, up to ten millimeters in total diameter." Later study and repeated replating in blood agar has shown that these four strains are alike in all respects. One of them, designated *Streptococcus* A-18 (Cow) B, has been described and photographed by Brown (1919). Apparently similar strains were isolated from horses by Jones (1919) who also published a photograph of one of them. Since then, many strains producing similar appearances in blood agar have been isolated from bovine and human sources. A brief description has been published (Brown, 1934).

The method for demonstrating the double-zones is as follows. To a tube of infusion agar, melted and cooled to 45 to 50°C., is added about 1 cc. of defibrinated blood and a loop of suitably diluted culture. The dilution of the culture should be such that there may be fewer than 100 colonies in the plate. After thorough mixing the inoculated medium is poured into a Petri dish and allowed to harden. The plates are incubated at 37°C. and then refrigerated. During the first 24 hours of incubation deep colonies with small zones of hemolysis appear and become larger as incubation is continued. Usually the zones of hemolysis have a rather poorly defined periphery but they will be found to be

clear immediately next to the colony and are therefore of the beta type. To determine this, the colony should be viewed in optical section under the 16 mm. objective of the microscope (figs. 1, 1a, 2, 2a). When the plate is refrigerated there appears a broad outer zone of partial hemolysis, resulting in the so-called "double-zone" (figs. 3, 3a, 3b). It is to be noted, however, that between the inner clear zone and the outer partly hemolyzed zone is a fairly distinct ring or zone of unhemolysed blood corpuscles. Throughout the zones there is no greenish or brownish discoloration such as appears in alpha zones. For comparison there are shown in figures 4, 4a, 5 and 5a, photographs of streptococci producing alpha and single beta zones in blood agar plates similarly incubated and refrigerated.

Although streptococci producing the above described appearance in blood agar were often isolated from mixed milk and the milk of cows with mastitis between the years 1915 and 1928, it was not until December of the latter year that a strain producing a similar appearance was obtained from a human source. Since then, over 100 strains have been isolated from human subjects during life and post-mortem. During life, strains were isolated from the throat, vagina, urine, a gangrenous leg and the blood stream. Many of those from the vagina and the throat were not associated with pathological conditions in these localities. Strains were isolated post-mortem from the heart blood, lung, ventricular fluid, peritoneal fluid and an abscess of the neck, often associated with other bacteria.

Various factors influence the readiness with which the double-zone appears, the degree of hemolysis in the outer zone, and the density of the intermediate zone of unhemolysed corpuscles. If the plates are incubated only 24 hours and then refrigerated, the colonies and the double-zones are smaller than when they are incubated 48 hours before refrigeration. Usually the outer zones do not appear until the plates have been refrigerated, although they are likely to be manifest if the plates are left at room temperature after incubation and rarely may appear during the latter part of the period of incubation.

Double-zones are not produced equally well in media made up

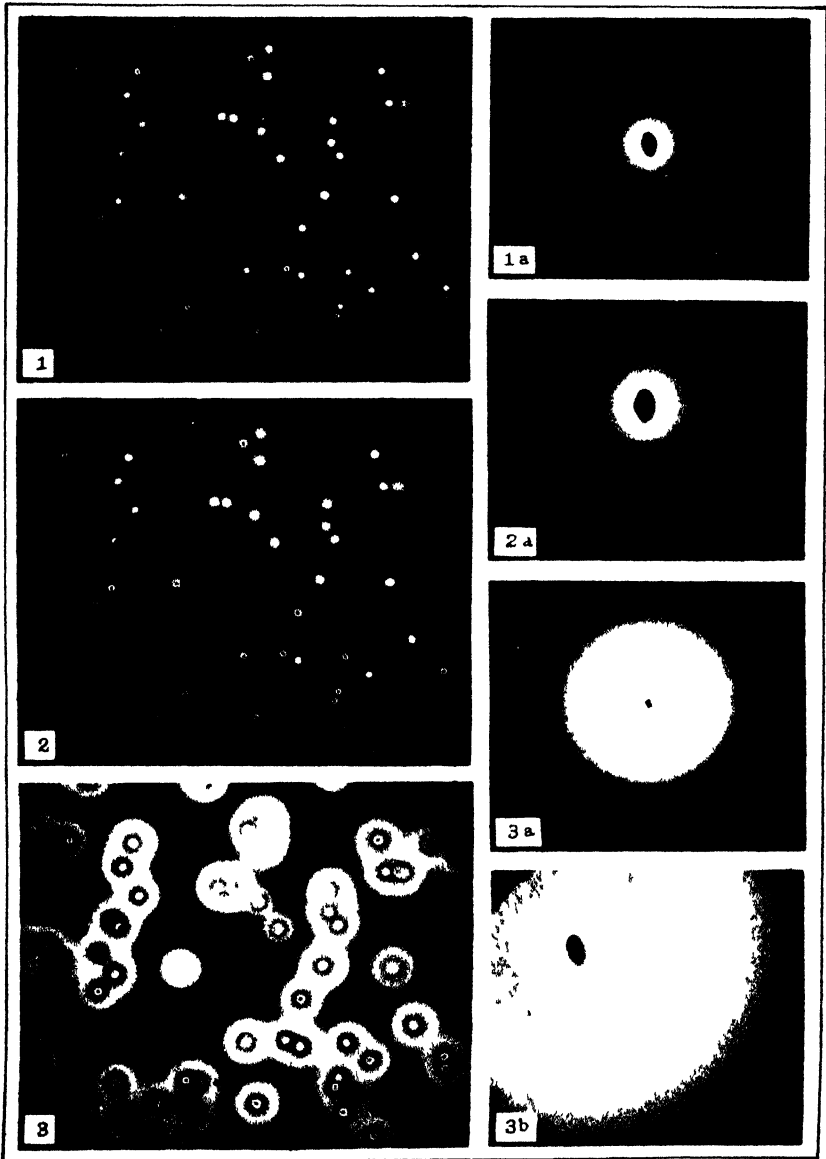


FIG. 1. A double zone beta hemolytic streptococcus in a blood agar plate incubated 24 hours, natural size. (Fig. 1a) Deep colony magnified in figure 1, magnified 37 diameters.

FIG. 2. The same plate as shown in figure 1 incubated 48 hours. (Fig. 2a) The same deep colony as shown in figure 1a.

FIG. 3. The same plate as shown in figures 1 and 2 refrigerated overnight. (Fig. 3a) The same colony as shown in figures 1a and 2a magnified 8 diameters. (Fig. 3b) The same colony, magnified 37 diameters.

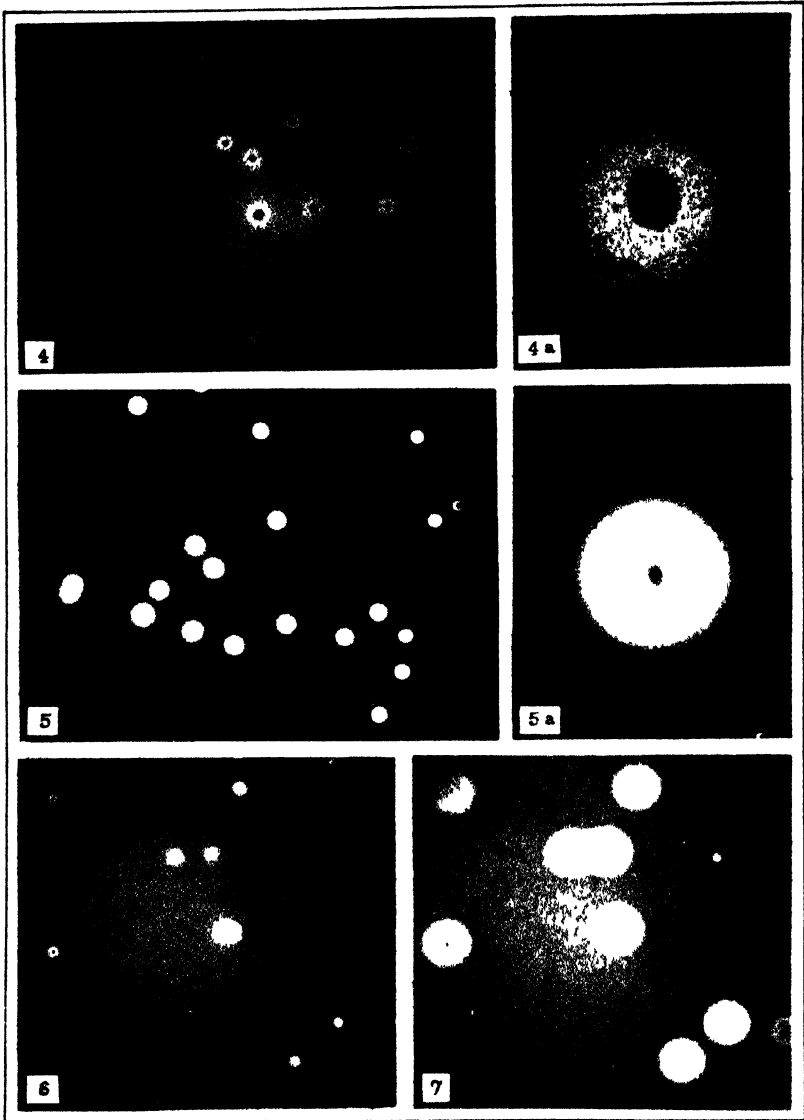


Fig. 4. An alpha streptococcus in a blood agar plate incubated 48 hours and refrigerated overnight, natural size. Fig. 4a. Deep colony marked in figure 4, magnified 37 diameters.

Fig. 5. A single-zone beta hemolytic streptococcus in a blood agar plate incubated 48 hours and refrigerated overnight, natural size. Fig. 5a. Deep colony marked in figure 5, magnified 37 diameters.

Fig. 6. A double zone beta hemolytic streptococcus in blood agar plate incubated 48 hours, with colony removed and placed onto the surface of the neighboring medium, natural size.

Fig. 7. The same plate as shown in figure 6 refrigerated overnight, natural size.

with various kinds of blood. A few bovine strains produce the zones most readily in horse blood but others, and most human strains, do not form double-zones in this blood. In the media used, rabbit blood served best for most strains, and was used routinely for this work unless otherwise stated. In a series of representative bovine and human strains human blood gave about the same results as rabbit blood; beef and sheep blood were less satisfactory.

With representative strains, pork infusion, veal infusion and beef infusion agars have been tried and each agar has been made with Wilson P, Witte, Bacto, Fairchild, Proteose and Neo-peptone. In all of these media and with either horse or rabbit blood all of the strains formed beta zones of varying size, but in certain of the media some of the strains failed to form double zones or some of the zones were atypical. A frequently encountered atypical double-zone was one which lacked the intermediate zone of unhemolysed corpuscles. In all three infusions typical double-zones were obtained most frequently when rabbit blood and Witte, Neo-, or Wilson P peptone were used.<sup>1</sup> Rarely a strain failed to form double-zones in one of these media but formed them in another. The reaction of the agar used was about pH 7.6. In extract agar made with Wilson P peptone all of a few representative strains formed double-zones with rabbit blood but not with horse or beef blood.

Although more or less atypical double-zones appeared in various agars at pH 6.5, none appeared at pH 6.0. Reactions more alkaline than pH 7.0 were optimal for typical double-zone formation.

Eight representative human and bovine strains were plated in duplicate in rabbit-blood veal infusion Witte peptone agar and incubated in Brown (1921, 1922) anaerobic jars. After incubation for 48 hours, one jar was opened and the plates were examined. All showed characteristic poorly defined single beta

<sup>1</sup> It is regretted that the results depended upon the use of proprietary products of incompletely known source, method of manufacture and composition. It is hoped that an investigation of peptones, now in progress, may help to remedy this situation.

zones of hemolysis similar to those produced by the same strains under aerobic conditions. The plates from this anaerobic jar were then refrigerated aerobically and within a few hours showed double-zones. The other anaerobic jar containing a duplicate set of plates was refrigerated without being opened after incubation for 48 hours. The double-zones formed in this jar were like those in the plates from the other jar refrigerated aerobically. It may be said, therefore, that these streptococci form double-zones after proper incubation and refrigeration under either aerobic or anaerobic conditions.

At present it is impossible to offer an adequate explanation of the double-zone phenomenon although certain observations may be suggestive. The alternate zones of hemolysis and unhemolysed corpuscles suggest Liesegang rings and this interpretation has been offered by Kortenhaus (1929) for somewhat similar zones formed by staphylococci and with respect to the alpha zones formed by pneumococci and alpha streptococci in blood agar. In an interesting series of papers Idzerda and van Everdingen (1932) have also indicated that these appearances in blood agar may be due to physico-chemical causes. Using hydrogen peroxide, mercury, or an electric current as hemotoxic agents they were able to produce zones of alternate rings of hemolysed and unhemolysed blood cells in sterile blood agar plates. The problem, however, is not as simple as it may seem.

A comparison of the hemolytic action of double-zone strains with that of the well known pathogenic hemolytic streptococci, such as *Streptococcus pyogenes* which may cause scarlet fever and septic sore throat, may be useful. After incubation of blood agar plates for 48 hours the double-zone strains form relatively small poorly defined zones of hemolysis; *Streptococcus pyogenes* forms larger, clearer, better defined zones. Broth cultures of double-zone streptococci vary in their ability to lysis 5-per-cent suspensions of rabbit blood cells within 2 hours at 37°C., whereas cultures of *Streptococcus pyogenes* produce complete laking. Apparently *Streptococcus pyogenes* is more hemolytic than the double-zone streptococci and yet when 48-hour blood agar plates

of *Streptococcus pyogenes* are refrigerated, little or no visible change occurs, double-zones do not appear. The comparison suggests (a) that different hemolytic substances are produced by these two groups of streptococci, or (b) that another factor or substance is involved.

When incubated blood agar plates of double-zone streptococci are placed into the refrigerator the double zones may appear in from 30 minutes to several hours. On one occasion no double-zones were present after refrigeration for 3 hours and were fully developed 30 minutes later. These observations suggest that during the period of incubation some substance has diffused outward from the colony and affected the fragility of the blood corpuscles in the surrounding medium and that laking of these corpuscles is brought about by exposure to lower temperature. Confirmation of this theory was obtained by removing by means of a cork-borer one of the colonies from an incubated blood agar plate and depositing the removed colony onto the surface of the neighboring medium (fig. 6). The plate was then refrigerated (fig. 7). The outer zone of partial hemolysis appeared not about the colony but about the area from which the colony had been removed.

An adequate explanation of the cause of the zone of unhemolysed blood corpuscles appearing between the inner and outer zones of hemolysis cannot be given. The more or less discolored rings or zones of corpuscles which appear in alpha zones of streptococci are thought by Hagan (1925), Leifson (1932) and others to be the result of methemoglobinization of corpuscles by hydrogen peroxide, but the unhemolysed corpuscles in the zones of double-zone beta streptococci are usually not discolored. That the phenomenon is not, at least quantitatively, the same as that appears with alpha zones is indicated by the following experiments. A blood agar plate was streaked with a culture of alpha streptococcus and immediately afterward this streak was crossed by similar streaks of beta streptococci. After 24 hours incubation the plate appeared as in figure 8. Streak I is of the alpha streptococcus and is greenish by transmitted light but without



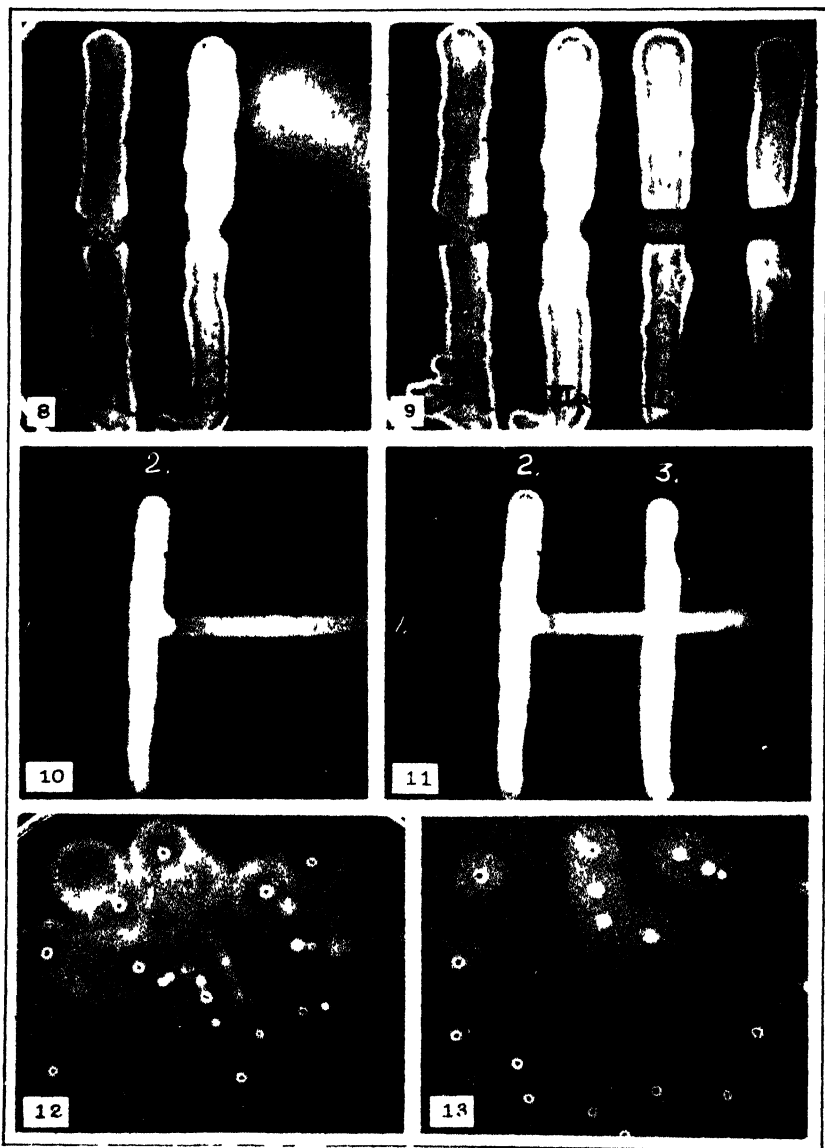


FIG. 8. A blood agar plate streaked with an alpha streptococcus (1) and then cross streaked with single zone beta streptococci (IIa and IIIa) incubated 24 hours, natural size.

FIG. 9. The same plate as shown in figure 8 after again cross streaking with single zone beta hemolytic streptococci (IIb and IIIb) and incubating for another 24 hours, natural size.

FIG. 10. A blood agar plate streaked with a double zone beta hemolytic streptococcus (1) and then cross streaked with a single zone beta hemolytic streptococcus (2), incubated 24 hours, natural size.

hemolysis. Streaks IIa and IIIa are of beta streptococci and show wide zones of hemolysis without discoloration. The hemolysis is not interrupted at the crossing of Streak I by Streaks IIa and IIIa. At the crossing, however, edges of the hemolysed streaks are indented where the corpuscles under Streak I had become "fixed" before the hemolysis had advanced that far. At this time Streak I was again crossed by streaks of the two hemolytic streptococci and the plate again incubated. After reincubation for 24 hours the result was as shown in figure 9. It is seen that in crossing Streak I, the hemolysis of Streaks IIb and IIIb is interrupted, the corpuscles underlying the growth of the alpha streptococcus having been fixed by it and rendered resistant to hemolysis by the beta streptococci (Brown, 1919). A similar experiment was conducted with a double-zone beta streptococcus in place of the alpha streptococcus (figs. 10 and 11). It was found that the unhemolysed corpuscles beneath the streak of the double-zone streptococcus were not rendered resistant to hemolysis by the beta streptococcus.

In view of the fairly common occurrence of double-zone beta hemolytic streptococci their lack of recognition may be due to several reasons. (1) It is necessary that blood agar pour plates be inoculated since double-zones often appear only about the deep colonies. (2) There must be a smooth even distribution of the blood in the agar and not too many colonies, preferably less than 100. (3) Plates should be incubated 48 hours at 37°C. and then refrigerated overnight since the double-zone usually appears only after refrigeration. (4) The kind and amount of blood used are important. Routinely we have used defibrinated rabbit blood and this has been satisfactory although some bovine strains produce double-zones more readily when horse blood is used. Horse

FIG. 11. The same plate as shown in figure 10 after again cross-streaking with the single-zone beta hemolytic streptococcus (3) and incubating for another 24 hours; natural size.

FIG. 12. A double-zone beta hemolytic streptococcus in a blood agar plate incubated 48 hours and then refrigerated; natural size. Photographed in March 1915.

FIG. 13. The same strain of streptococcus as shown in figure 12. Blood agar plate incubated 48 hours and then refrigerated; natural size. Photographed in April 1936.

blood is not satisfactory for many strains of human origin. The amount of blood in the agar should be between 5 and 10 per cent or about 1 cc. of blood to 12 cc. of agar which, when poured into a 10-cm. Petri dish makes a layer of medium about 2 mm. thick. (5) The agar used has been a meat infusion agar with a reaction of about pH 7.6. Pork, beef and veal infusions have been tried as have also various peptones. Veal infusion with Witte peptone has given the most uniformly satisfactory results. Other combinations give good results with certain strains but may fail to allow the production of double-zones with others. Further experiment is necessary to determine whether extract agar may be used with equally satisfactory results.

That others may have observed the double-zones here described is indicated by the following quotations. "The streptococcus on the plates which I show you this evening was isolated by Dr. Baumann from the blood of a case of phlebitis and paraphlebitis of the leg. On the blood agar plates it was noticed that after 24 hours the organism was surrounded by a clear area. Surrounding this clear area the blood on the plate was darker in color than on the rest of the plate. After forty-eight hours a second clear ring appeared outside of this area of darkened blood. In one observation the process extended so that there were three clear concentric rings about the streptococcus colony." (E. Libman, 1905). Because of the lack of technical details it is impossible to decide whether the appearance described was alpha or beta. This is rendered more doubtful by a similar description of a pneumococcus colony by the same author in which he says "next to the colony was a clear ring, surrounding this was a ring of somewhat darkened blood, outside of which there was again a clear ring." (E. Libman, 1905). We know that the pneumococcus forms an alpha zone, often with considerable hemolysis. We must reemphasize that unless the deep colony in blood agar is studied under the microscope it is unsafe to make a diagnosis of alpha or beta zone.

Taylor and Wright (1930) describe certain strains of hemolytic streptococci isolated from the human vagina which apparently formed double beta zones in blood agar plates left at room tem-

perature or refrigerated after incubation. "In horse blood agar little or nothing happens except a slight lightening in colour, barely amounting to more than a haziness, at the periphery of the haemolysed zone. In rabbit-blood agar a similar zone develops much more commonly, at first as a wide belt, rather light in colour, surrounding the haemolysed zone next to the colony. Later, this belt tends to differentiate into an outer haemolysed zone and an inner usually quite narrow unhaemolysed part. Further incubation for 48 hours leads simply to greater emphasis of the outer zone of haemolysis and sometimes to disappearance of the intermediate unhaemolysed zone. . . . There is one strain described by Brown and illustrated in Figures 3-6, Plate 4, of his monograph illustrating a change not unlike what is here described - the formation on horse blood agar of a certain discolouration beyond the haemolysed area." This description agrees with our observations of the double-zone beta streptococci of which the one referred to by Taylor and Wright was one of the first that we isolated. The reference to "a certain discolouration beyond the haemolysed area" is an error. We were careful to state on page 30 of the monograph "There is no discoloration," meaning by this that the remaining corpuscles were red, not greenish or brown.

Stableforth (1932) states that all of his strains of Group I, type b were somewhat peculiar in regard to hemolysis. "After 24 or 48 hours incubation a large part of the colonies were surrounded by a narrow but clear zone of hemolysis outside which was produced a greenish ring similar to that found immediately around the colony of an alpha strain. Outside this ring of fixed cells was another in which hemolysis was again complete. This appearance does not exactly correspond to Brown's description of an alpha prime ( $\alpha_1$ ) nor in fact to any of Brown's categories. There was, moreover, another character which precluded the placing of these amongst the alpha ( $\alpha_1$ ) group, viz., the marked lysin production in fluid media. It should be added that in strains of no other type was this strong double-ringed tendency observed." It seems quite likely that Stableforth observed the double-zone beta appearance although the blood which he used

(ox-blood) has in our hands not been favorable for this purpose. The greenish discoloration which he mentions may have been due to the medium used. We have noticed it when sheep blood was used and it is favored by some brands of peptone. He did not recognize the similarity of the appearance which he describes to the illustrations published by the author in 1919 (plate 4, figs. 3, 4, 5 and 6).

Hare and Colebrook (1934) describe strains of streptococci from bovine mastitis and from the human vagina which doubtless belong to the group of double-zone beta hemolytic streptococci. They used horse-blood agar and state that "the zone of hemolysis is only complete in the immediate neighbourhood of the colony and outside this is a hazy zone consisting of islands of unhemolysed cells in a hemolysed matrix. Brown (1919) figures this appearance in his monograph (plate 5, figs. 1, 2, 1a and 2a)." In defibrinated horse blood the corpuscles normally are agglutinated. The "islands of unhemolysed cells" noted by Hare and Colebrook are doubtless due to the fact that the larger aggregates of cells are less readily hemolysed than others. Continuing, "With some strains from the human vagina and from the cow's udder this partially hemolysed zone may be quite wide and sharply demarcated from the inner zone of complete hemolysis." These were probably what we call double-zones similar to those illustrated in plate 4, figures 3, 4, 5 and 6 of the monograph (Brown, 1919). As stated above, some strains which produce double-zones in rabbit blood agar may not do so in horse-blood agar. This has been found to be true of the strain illustrated in Plate 5 (Brown, 1919) and referred to by Hare and Colebrook.

Apparently the ability of these strains of streptococci to form double-zones is not a transient character, but is quite stable. We have not noted its disappearance from any of the more than 150 strains which we have in cultivation. The first strains were isolated by Smith and Brown (1915) in 1913. In figure 12 is shown a blood agar plate of one of these strains photographed in 1915. In figure 13 is shown a photograph of the same strain plated in 1936. Both show double-zones. Minor differences in the appearance of the zones in the two photographs may be due

to unrecognized variations in the media, but are partly due to differences in the methods of photography.

#### SUMMARY

The streptococci of a certain group of beta-hemolytic streptococci form double zones in blood agar plates refrigerated after incubation. This characteristic persists after long periods of cultivation of these strains. These streptococci are from human and animal sources. In a forthcoming publication the cultural characteristics of double-zone beta hemolytic streptococci will be described with some reference to their serological grouping, source and pathogenic significance.

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# DISSOCIATION IN *BACILLUS SALMONICIDA*, WITH SPECIAL REFERENCE TO THE APPEARANCE OF A G FORM OF CULTURE

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It is the purpose of this communication to record certain dissociative phenomena in *Bacillus salmonicida* Emmerich and Weibel (1894). This microorganism, the etiological agent of an epizootic disease ("furunculosis") of salmonid and other fishes, came to the attention of this laboratory through having been identified as a cause of disease amongst the cutthroat trout and Rocky Mountain whitefish in British Columbia (Duff, 1932, Duff and Stewart, 1933). Other work on *B. salmonicida* is adequately reviewed in the Reports of the Ministry of Agriculture and Fisheries, Great Britain (1929, 1933, 1935).

Stock cultures of various British Columbia strains of *Bacillus salmonicida* have been carried in this laboratory since August, 1931. Following 6 or more months of artificial culture a marked loss of pathogenicity occurred in all strains. Furthermore a gradual change occurred in the appearance of colonies upon nutrient agar. Immediately after isolation of a strain from an infected fish, the colonies appeared glistening, convex, and translucent. Stock cultures about 6 months after isolation gave rise to colonies more strongly convex, distinctly opaque, and cream-colored. Changes in pathogenicity and colony formation were not accompanied by any change in the ability of the colonies to produce the diffusible brown pigment typical of the species. Occasional loss of pigment-producing ability was noted in some stock cultures, but the ability could be recovered by rapid serial



plating, and in any case this variation could not be correlated with the dissociative activities about to be described. Such observations called for a more detailed investigation into the dissociative potentialities of the species, and to this end it was decided to attempt to enforce dissociation upon stock cultures by methods already applied successfully by other workers to other bacterial species (Hadley, Delves and Klimek, 1931).

Three strains of *B. salmonicida* were selected:—Elk River 3 (ER 3), Cultus Lake 6 (CL 6), and Stanley Park 2 (SP 2). From each of these a single-cell strain was derived by a method previously described (Duff, 1929). Cultures of each single-cell strain possessed the regular cultural and biochemical characters proper to the species, and gave rise to colonies on nutrient agar of the type described above for older stock cultures. Each strain was then subjected to serial subculture at 48-hour intervals, at 19 to 21°C., in the following media:—

- (a) Nutrient broth,<sup>1</sup> pH 7.0
- (b) Nutrient broth, pH 8.0
- (c) Nutrient broth, pH 7.0 plus 0.25 per cent lithium chloride
- (d) Nutrient broth, pH 7.0 plus 0.1 per cent phenol

Transfers consisted of 3 drops from a Pasteur pipette. At the moment of transfer, a drop of the culture was also spread upon the surface of a nutrient agar plate by means of a glass spreader.

No changes in colony appearance occurred in any strain during transfer in plain nutrient broth (either at pH 7.0 or pH 8.0) up to the fortieth transfer, when these series were discontinued. Definite dissociation into two clear-cut colony types, with intermediate forms, occurred in all three strains under the influence of lithium chloride broth and of phenol broth.

#### DISSOCIATION IN PHENOL BROTH SERIES

The behavior of each strain under the influence of phenol broth was, except for minor details, identical; the description given below of dissociation in the SP 2 strain may be regarded as typical of all three single-cell strains. Nutrient agar spread plates made

<sup>1</sup> Bacto Dehydrated, Digestive Ferments Company.

from 3 drops from the 48-hour growth in the first and second serial tubes gave rise to 30 to 40 colonies of relatively great size (5 to 8 mm. diameter in 96 hours) in place of the several hundred colonies of normal size (about 2 mm.) arising on a plate when spread with the same amount of inoculum, of equal turbidity, from plain broth or from lithium chloride broth. These giant colonies produced the typical diffusible brown pigment with marked intensity. Platings from the third tube of the series yielded similar giant colonies, and in addition some 20 colonies per plate, which had a highly convex center of the usual consistency, surrounded by an annulus of thin, flat transparent growth with entire edge. Platings from the fourth serial tube yielded again the colony types described for tube three, and in addition numerous colonies, approximately 1.5 mm. diameter, which appeared to have a roughened surface. Examination with a hand lens showed that the roughness was due to the presence of large numbers of minute secondary papillae on and below the surface of each colony.

There were thus three abnormal colony types arising out of passage of the organism through phenol broth—"giant," annular, and papillated colonies. Further platings on nutrient agar were made from the "giant" colonies, from the central body and annulus of the annulated colonies, and from the papillae of the papillated type. It proved impossible to obtain a satisfactory sample of the body of the latter colony type free from papillae, but the papillae themselves could be touched at the center with a fine needle and so obtained in presumably pure form. Material obtained in this way was rubbed into a few drops of sterile saline in a sterile tube, and plates were spread with the suspension. Nutrient agar plates spread from the "giant" colonies showed an immediate reversion to the normal stock colony type. Plates spread from the body and periphery of the annulated colonies also showed immediate reversion to the normal stock type.

In contrast to the complete reversion of these variants, platings from papillae showed colonies of the original (stock culture) type, but in addition approximately equal numbers of a new colony form, together with a number of colonies of an intermediate type.

Selected colonies of the two distinct types when replated gave rise only to colonies of the same description as that of the parent colony, whereas platings from the intermediate type of colony always showed a further breaking up into the two major and the intermediate colony types.

#### DESCRIPTION OF DISSOCIANTS

The colonies resembling those of the original stock culture were, after 48 hours incubation, opaque, strongly convex and cream-colored. They possessed a physical consistency which permitted the entire colony to be pushed about on the surface of the agar with a cool loop, without disturbing the form of the colony or leaving immediate traces of its passage on the surface. When seeded to nutrient broth, growth appeared in the form of light floccules, having a tendency to settle rapidly to the bottom of the tube leaving the supernatant fluid clear. The morphological, fermentative, and pigment-producing characteristics of this culture type were those characteristic of *B. salmonicida*. The new colony type was entirely different from that of the initiating culture, and corresponded more closely with the kind of growth observed immediately after isolation of the organism from diseased fish. Colonies on agar at 48 hours were translucent, only slightly convex, and showed a slight but definite bluish-green color by transmitted light. The consistency of these colonies was butyrous, so that a loop pushed against them would destroy the structure and smear the growth over the agar surface. Growth in broth appeared as a uniform turbidity, with little sedimentation except in cultures 72 hours or more in age. Morphological, fermentative, and pigment-producing characteristics were again those typical of *B. salmonicida*.

#### PATHOGENICITY OF DISSOCIANTS

The goldfish (*Carassius auratus*) was utilized as a test fish, having previously given consistent results in our work in testing the pathogenicity of freshly isolated strains of *B. salmonicida*. Three strains of the cream-opaque type of colony, and three of the green-translucent, were selected for testing. Each strain was

inoculated into 3 goldfish. The fish were inoculated intraperitoneally with 0.1 cc. of a No. 4 McFarland suspension made from a 48-hour broth culture of the strain in question.

In the case of all three strains derived from the green-translucent colony type, all fish died within 9 days, with lesions typical of the disease. *B. salmonicida*, of the same colony phase as was used for inoculation, was recovered from the kidneys and heart blood of the fish. In two of the strains derived from the cream-opaque type of colony, the fish survived for over 30 days without sign of illness. In the remaining strain, 2 fish survived for 30 days, the third died in 20 days, and a mixed culture of *B. salmonicida* (cream-opaque type) and *Pseudomonas pyocyaneus* was recovered from the blood and kidney. The fish showed none of the lesions typical of "furunculosis."

#### DISSOCIATION IN LITHIUM CHLORIDE BROTH SERIES

This incitant, just as was the case with phenol broth, exerted a remarkably uniform influence upon the three strains of *B. salmonicida* involved. Under its influence the same stable variants arose, although the time required for their production was much longer than in the phenol broth series. Moreover in the lithium chloride broth series the stable variants appeared directly upon plates made from certain tubes in these series, instead of being derived secondarily from papillated colonies, as was the case with phenol broth.

Table 1 records the fact that dissociation commenced in one strain in the 15th, and in the other two strains in the 17th serial subculture. Up to the time of the first appearance of dissociation, progress was followed by making only one spread plate from the current serial tube. At the first sign of dissociation the sampling from each tube was increased to three spread plates. The percentage distribution of dissociants recorded in the table is therefore based on a count of only one plate for the first day on which dissociation was observed, and thereafter on a count of three plates. The variants recorded in the columns R and S of the table corresponded exactly with the description already given for these types as derived from phenol broth, the cream-colored,

opaque colony type being recorded in column R, the greenish, translucent type under column S, and intermediate types under column RS. It will be noted that in each strain, the original colony type gradually gave way over a period of days to more and more of the new form. As with the variants recovered from

TABLE 1  
*Dissociation in lithium chloride broth series*

TUBE NUMBER	STANLEY PARK 2				CULTUS LAKE 6				ELK RIVER 3			
	100 per cent			G	100 per cent			G	100 per cent			G
	R	S	RS		R	S	RS		R	S	RS	
1 to 10	100	—	—	—	100	—	—	—	100	—	—	—
11	100	—	—	—	100	—	—	—	100	—	—	—
12	100	—	—	—	100	—	—	—	100	—	—	—
13	100	—	—	—	100	—	—	—	100	—	—	—
14	100	—	—	—	100	—	—	—	100	—	—	—
15	100	—	—	—	60	20	20	—	100	—	—	—
16	100	—	—	—	61	22	17	—	100	—	—	—
17	65	24	11	—	60	25	15	—	71	26	3	—
18	61	32	7	—	57	25	18	—	60	26	14	—
19	44	38	18	—	48	32	20	—	48	40	12	—
20	17	25	58	—	25	60	15	—	15	50	35	—
21	11	71	18	—	21	60	19	About 3000	16	57	27	110
22	10	49	41	About 1500	11	65	20	—	10	83	7	—
23	8	90	2	About 1500	5	86	9	—	7	85	8	—
24	7	90	3	—	5	90	5	—	7	78	15	—
25	8	88	4	—	5	91	4	—	4	86	10	—
26	—	—	—	—	—	—	—	—	4	88	8	—
27	—	—	—	—	—	—	—	—	5	88	7	—
28	—	—	—	—	—	—	—	—	5	90	5	—
29 to 32	—	—	—	—	—	—	—	—	—	—	—	—

papillated colonies in the phenol broth series, replating of selected cream-opaque green translucent colony types produced cultures many of which remained constant in character even after maintenance in the laboratory for over a year.

The pathogenicity of the two colony types derived from lithium chloride broth was tested in the same manner as for the phenol

broth derivatives, and with the same result--the green-translucent type proving pathogenic, the cream-opaque non-pathogenic.

Two methods of enforcing dissociation upon single-cell stock strains of *B. salmonicida* have therefore each yielded the same two cultural types. One, the cream-opaque type, is non-pathogenic, more stable on prolonged culture, produces flocculent growth in broth, and friable colonies on agar. The other, the green-translucent, is pathogenic, producing on inoculation the typical lesions of the disease, less stable on prolonged culture, gives a homogeneous clouding in broth, and butyrous colonies on agar. These cultural types of *B. salmonicida* thus show respectively the major characteristics of the two classical dissociant forms, rough (R) and smooth (S), first described by Arkwright (1921) for members of the colon-typhoid dysentery group and since recorded by others for many species of microorganisms. They seem naturally to fall into the standard classification as the Rough and Smooth forms of *Bacillus salmonicida*.

It is to be noted that the laboratory methods used to enforce dissociation on *Bacillus salmonicida* were applied to the non-pathogenic, R phase of the microorganism, and resulted in the production of the pathogenic, S phase. While the general tendency amongst bacteriologists seems to have been to regard the R phase of a species as the more stable stage, reverting with difficulty or not at all to the S type, several works may be quoted which record the production of S from R forms in a variety of microbial species. Jordan (1926) records R to S transformation in *Salmonella schottmuelleri*. Griffith (1928), Neufeld and Levinthal (1928), Reimann (1929), and Dawson (1930) were able by means of an *in vivo* technique to transform R forms of the pneumococcus into the S forms of the homologous or heterologous types. Dawson and Sia (1930) and Alloway (1932) achieved the same results by entirely *in vitro* methods. Petroff and Steenken (1930) record the R to S transformation in certain strains of *Mycobacterium tuberculosis*. Reed and Rice (1931) effected the transformation of a bovine strain of this organism from R to S by rapid transfer through alkaline fluid media. Hadley, Delves and Klimek (1931) record R to S dissociation in the Shiga dysen-

tery bacillus when grown in certain lithium chloride broth and pancreatin broth series. Torrey and Montu (1936) produced early and final R phases from an S culture of a virulent strain of *Escherichia coli*. The early R form, although avirulent, readily reverted to the virulent S form. Spicer (1936) induced R to S dissociation in hemolytic streptococci of scarlet fever and erysipelas origin, by growing her cultures in beef-heart broth for 15 to 19 days. Wickerham and Fabian (1936) effected R to S transformation in certain yeasts by growth in malt extract agar.

#### APPEARANCE OF THE G-TYPE CULTURES

During the period when dissociation of the R to S type was occurring in the lithium chloride broth tubes, another colony form appeared on one or two occasions in each series, upon all of the triplicate plates for each occasion. This phenomenon is recorded under column G of table 1. These colonies appeared in large numbers in the case of SP 2 and CL 6 strains, and in much smaller numbers in the ER 3 strain. They were very small, (0.2 to 0.3 mm.), transparent, and perfectly circular, and only became visible after 72 hours incubation. Slide preparations from these colonies showed a very small, Gram-negative coccoid microorganism (averaging  $0.3\mu \times 0.5\mu$ ) appearing in clumps in a manner similar to the arrangement of the larger *B. salmonicida*. Colonies of this third culture type were picked to slant agar for future investigation and will be referred to later.

The 26th<sup>th</sup> lithium chloride broth tube in the SP 2 series, although it had received the usual inoculum (3 drops of 48-hour culture of the usual turbidity) remained apparently sterile after 2 days' incubation. Drops from this tube spread upon agar showed no visible growth even after 10 days' incubation. The same phenomenon—a sudden apparent auto-sterilization of the culture—occurred in the 26th tube in the CL 6 series, and in the 29th tube of the remaining strain. It did not seem reasonable to assume that the strains had died off as a result of continuous exposure to an unusual and unfavorable substance—lithium chloride—for the reason that growth did not become progressively poorer from tube to tube of the series, but maintained its usual

vigorous rate up to and including the tube immediately before that which remained apparently sterile. However, had it not been for knowledge of the similar experiences of Hadley, Delves and Klimek (1931) when dealing with serial subculture of the Shiga dysentery bacterium and other species, the present series would at any rate have been terminated at this point. With this previous work in mind, however, serial transfers were carried on as usual in all three strains up to the 32nd tube, with transfers of the usual amount of fluid, after the usual incubation time, from tube to tube in the series and as usual to agar plates.

During this section of the transferring routine, none of the tubes involved (Nos. 26 to 32 in two of the series and Nos. 29 to 32 in the third) showed any signs of growth visible to the naked eye. In the first "sterile" tube of each series a faint turbidity had been present due to the usual heavy inoculum (3 drops) from the previous tube. Examination of these tubes after 48 hours incubation failed to show any cloudiness at all, even when the tubes were gently agitated. As this point was of some interest, six other lithium-chloride broth tubes were inoculated in the same manner and amount from the last tube of each series to show growth. To three of the tubes, 0.5 per cent formalin was added. The slight turbidity due to the inoculum remained indefinitely in the formalinized tubes, but again disappeared in 48 hours in the untreated tubes. So far as the remaining "sterile" tubes in each series were concerned, none of these, with the exception of No. 30 in the Stanley Park series, ever displayed the faintest turbidity, although they were held for a period of over 2 months.

The phenomenon of sudden cessation of obvious growth in three different series, following closely upon a dissociative reaction involving two chief variants and a possible third variant (the small colonies), was analogous to the similar findings of Hadley, Delves and Klimek with regard to the Shiga dysentery bacterium and other species. In the series recorded by these workers, apparently sterile tubes were followed by tubes yielding their G-type of culture. In other portions of the same paper, the so-called "washed-plate" technique of Hauduroy (1927) is employed to recover visible G colonies from Berkefeld filtrates of certain



cultures. Although, as already recorded, visible growth was not present in the later lithium-chloride broth tubes of the series under consideration, it was thought possible that the invisible phase of the G-type culture might be present, and in order to attempt a demonstration of its existence, it was decided to apply the Hauduroy technique to the contents of these tubes. Since triplicate primary platings had been continued as a matter of routine from all the apparently sterile tubes in the three *B. salmonicida* series, it was possible to select plates for the Hauduroy procedure from those which were not only sterile in respect to the tubes from which they were plated, but which were also perfectly clear of any subsequent contaminations from the air. No plate showing air contamination could be used in any case, owing to the nature of the Hauduroy technique, but an additional check on proceedings was kept by identifying any apparently contaminating colonies which did appear on a few plates. None of these plate contaminants showed any resemblance either to *B. salmonicida* or to the G-type colonies which eventually arose from the washed-plate treatment.

The Hauduroy process as carried out in this laboratory consisted in placing upon the surface of a primary agar plate (that is, one spread direct from a "sterile" tube in a series and showing no growth after incubation) about 10 drops of sterile broth from a sterile Pasteur pipette. Just before drawing up the sterile broth into the pipette, the tip of the latter was bent in the flame to a slight angle. This allowed the pipette, after delivery of the broth, to be used as a spreader to distribute the fluid over the surface of the agar. A portion of the fluid was then collected in the pipette, transferred to the surface of two fresh sterile agar plates, and spread as before. After 72 hours' incubation at room temperature, providing no G-type colonies appeared, one of the two secondary plates was selected and the process repeated as often as required. The Hauduroy technique was applied to plates from all 7 "sterile" tubes in the Stanley Park 2 series, from tubes 26 and 29 in the Cultus Lake 6 series, and from tubes 25, 29 and 30 in the Elk River 3 series, with the following results:—

- SP 2*. No. 26: Tube seeded 5-7-35. Washed-plate series started 27-8-35, carried to 4th plating. No appearance of G-type colonies. Another washed-plate series from same tube started 20-9-35, carried to 6th plate. Negative.
- SP 2*. No. 27: Washed-plate series started 12-8-35, carried to 4th plating. Negative. Another washed-plate series started 20-9-35, carried to 6th plating. Negative.
- SP 2*. No. 28: Washed-plate series started 27-8-35. On the 4th washed plate (i.e., 5th plate from tube) at 48 hours' incubation large numbers of minute colonies became visible under a hand lens. In 96 hours these became visible to the naked eye, and numbered approximately  $1 \times 10^6$ . The colonies varied from 0.1 mm. to 0.2 mm. in diameter, and the size did not increase on further incubation. They were transparent by transmitted light, grayish by oblique lighting, slightly convex and perfectly circular.
- Sp 2*. No. 29: Washed-plate series started 27-8-35. The 4th washed plate yielded approximately the same number of colonies of the same description.
- SP 2*. No. 30: This tube behaved differently from all other "sterile" tubes. It remained apparently sterile from 9-7-35 to 19-8-35, during which period the tube was not opened or otherwise disturbed. On 20-8-35 a faint clouding appeared, increasing slowly during the following 7 days, when the turbidity approximated that of McFarland's No. 1 standard. Film preparations made on 27-8-35 showed minute Gram-negative coccoid bodies (averaging  $0.3 \mu \times 0.1 \mu$ ) arranged in clusters. Agar plates spread from the tube and incubated 72 hours showed a thin, somewhat fluorescent film over the thickly spread portion of the plate, and minute transparent colonies on the remainder of the surface. These colonies were exactly like those recovered from tubes 28 and 29 by the washed-plate technique.
- Sp 2*. No. 31: Washed-plate series started 27-8-35. The third washed plates yielded each about 3000 colonies of the same description as above.
- Sp 2*. No. 32: Washed-plate series started 27-8-35. The fifth washed plates yielded each about 1300 colonies of the same description.
- CL 6*. No. 26: Washed-plate series started 27-8-35, carried to 6th plating; negative. Another series started 20-9-35, carried to 6th plating; negative.
- CL 6*. No. 29: Washed-plate series started 19-8-35; the 4th set of washed plates yielded about 3000 colonies of the same description as those recovered from the *SP 2* series.
- ER 5*. No. 25: Washed-plate series started 12-8-35, carried to 5th plating; negative. Another series started 20-9-35, carried to 6th plating; negative.
- ER 5*. No. 29: Washed-plate series started 27-8-35. The 4th set of washed plates each yielded approximately 2000 colonies identical in appearance with those recovered from the *SP 2* and *CL 6* tubes.

Agar slant cultures were made from the colonies, growth on these appearing as a flat transparent film on moist slants, but tending to form discrete colonies on dried agar. Broth cultures grew poorly if seeded directly from the plates on which the colonies first appeared. Subcultures to broth after a few transfers

on agar gave rise to uniform clouding in 72 hours, followed by the accumulation of a visco-granular sediment which attained considerable bulk in tubes 10 days or more old. When seeded into nutrient broth containing glucose, maltose, mannitol, lactose and sucrose, growth occurred as in plain broth but without fermentation of any of these carbohydrates.

A strain selected from each of the 3 series was subjected to a pathogenicity test. Broth cultures equal in density to No. 2 McFarland Standard were employed. For each strain, 3 goldfish received 0.25 ml. of the culture intraperitoneally. The fish were kept under observation for 3 months, during which time no illness was apparent and no lesions developed. The fish were then sacrificed and cultures taken upon agar from peritoneal washings, kidney, and heart blood. No microorganisms were recovered from any fish, except in one case when a pure culture of *Pseudomonas pyocyaneus* appeared from a kidney culture.

Reference must now be made to the small colonies which appeared on plates of the three lithium-chloride broth series at certain times during the phase of dissociation of the parent culture (table 1, column G). These had been transferred to agar for future examination. Replatings showed that they were identical in all respects with the small colonies which have just been described as having been recovered by special methods from apparently sterile tubes later in each series. These earlier cultures were again inert as to carbohydrate fermentation and pathogenicity, and were morphologically identical with those obtained from "sterile" tubes.

The appearance, therefore, of a minute colony form during dissociation, and later from apparently sterile tubes, in lithium chloride broth series of *B. salmonicida*, corresponds most closely with the appearance, under similar conditions, of the G-type variants of the Shiga dysentery bacterium and of other species recorded by Hadley, Delves and Klimek. (G-forms for other bacterial species have since been recorded by numerous workers, including Koser and Dienst (1934), Hoffstadt and Youmans (1932), Hadley and Carapetian (1933), Rettger and Gillespie (1933), Roos, Réichel and Clark (1934), Poe (1934), Kopeloff

(1934) and Chinn (1936). It was therefore probable that the new cultural form obtained above was the corresponding G-type of *Bacillus salmonicida*.

#### CONTROL METHODS

Space does not permit a full discussion of the vexed question of "contamination" in relation to the appearance of the G-type of culture. At this stage of the findings the criticism will no doubt be made that the G-culture, supposed to be a variant of *B. salmonicida*, may have found its way from the air or from some other source into the several tubes of the lithium chloride broth series from which it was recovered. It might also be claimed that the exercise of the Hadley washed-plate technique, subject as it may be to contamination during a long series of plate-to-plate transfers, would give ample opportunity for the entry of foreign microorganisms into the systems in question. So far as air contaminations are concerned, it may be said that all the control measures adopted by Hadley, Delves and Klimek have been utilized in this work. For every three experimental washed-plate series, a control washed-plate series was carried out with sterile lithium chloride broth as the initial inoculum. Each transfer, on the controls as in the experimental series, was made to two fresh sterile agar plates. While contaminating microorganisms, either *Staphylococci*, *Sarcinae*, *Bacillus subtilis*, *Actinomyces* or molds, occasionally appeared on plates in the series, they never appeared on both duplicate plates, and it was always possible to continue the series by washing from the uncontaminated plate. Plates from which washings had been taken were retained for a further 3 days to make sure that contamination had not occurred during the actual manipulation. No colonies even remotely resembling the G-types recovered from the experimental series, were ever observed in the control series. Moreover, plates were exposed to the air at weekly intervals during the procedure, and while these picked up the usual air contaminants, they never yielded the minute, transparent colonies, maintaining a uniform size on continued incubation, which characterized the tubes recovered in the experimental series.

RECOVERY OF TYPICAL *B. SALMONICIDA* FROM G-VARIANTS

Conclusive proof of the relationship of the G-type cultures with the presumed parent strains of *B. salmonicida* rests however on the fact that typical R strains of the species have been recovered from G-type cultures derived from all the three strains of the organism (ER 3, CL 6, SP 2) which were subjected to serial transfer in lithium chloride broth. In spite of the fact that the G-strains in question had not been passed through Berkefeld candles (as in some series of Hadley, Delves and Klimek) their reversion to the parent R form was an exceedingly slow process. Three strains of G-culture were selected from those which had been derived from the ER 3, CL 6, and SP 2 strains of *B. salmonicida*. These G-strains bore the numbers GE 17, GC 7, and GS 22 respectively. At the time when attempts at reversion were begun, these strains had existed as typical G-cultures for two months since isolation, without showing any increase in colony size or change in morphology, and without having developed any ability to ferment glucose, maltose and mannitol. Reversion was attempted in the following ways:

(1) Serial subculture in plain nutrient broth.<sup>2</sup>

(2) Serial subculture in nutrient broth containing 0.25 per cent lithium chloride.

(3) Serial subculture in lithium chloride broth followed by serial subculture in tubes and plates of nutrient broth and agar containing 3 per cent of a mold filtrate.

(4) Observation of aged G-cultures.

In series (1) transfers were begun on 5-11-35 and were made every third day. This was the period required for the production of moderate turbidity in tubes seeded with G-cultures, at room temperature. The serial transfers were carried on for 8 months, with platings on agar at every third transfer. No change occurred in the characteristics of the three G-strains. Series (2) was carried for 8 months. For the first 3, no change in characteristics was apparent. During the next 2 months a more luxuriant and rapid growth in broth developed, and the

<sup>2</sup> Bacto dehydrated.

colonies attained a size about midway between the G size and those of normal 48-hour *B. salmonicida*. The organisms however were still inert as to carbohydrate fermentation. This state continued for all strains during the 6th and 7th months. During the 8th month some of the colonies from strains GE 17 and GS 22 again showed an increase in size, although the greater number of colonies on any given plate retained the intermediate size. Colonies from the remaining strain (GC 7) remained in the intermediate-size stage without further progression during the 8 months of transfer in lithium chloride broth. Replatings on agar from the larger colonies from strains GE 17 and GS 22 gave rise to colonies of 2 mm. diameter, and of the exact description already given for the R colony of *B. salmonicida*. Cultures from these colonies after several transfers began to produce the diffusible brown pigment characteristic of the parent organism, and when tested on carbohydrates fermented glucose, maltose and mannitol and failed to ferment lactose and sucrose--the reactions proper to the parent cultures. The morphology of the cultures was identical with that of *B. salmonicida*.

When tested on goldfish, the strains were non-pathogenic. This was to be expected, since the cultures bore all the characteristics of the R or non-pathogenic culture. However, it was considered necessary to attempt completion of the whole cycle and to try to obtain, from these cultures, a strain which would produce the typical disease. Accordingly, dissociation was again enforced upon the recovered R strains of strains GE 17 and GS 22 by the phenol broth method already described. S strains were obtained after the 4th to 6th transfer in 0.1 per cent phenol broth. These S cultures produced the typical lesions of the disease in goldfish, resulting in the death of the fish at periods varying from 6 to 12 days. *B. salmonicida* S type was recovered in pure culture from the kidneys and heart blood of the victims.

Procedure (3) was adopted in view of the following observations. Several mold colonies had appeared on some of the plates from serial transfers after these plates had been opened for inspection or had been left in the laboratory for a week or more. It was noticed that the G-colonies in the neighborhood of the

molds attained a large size compared with those on other parts of the plate. This suggested that some growth-stimulating factor of a diffusible nature was being elaborated by the mold. The latter was therefore isolated in pure culture, and proved to be a variety of *Aspergillus glaucus*. When the mold was inoculated upon plates seeded with G-cultures, the same phenomenon of growth stimulation was again observed. The idea naturally arose of adding a sterile filtrate of mold growth to nutrient broth, and using this medium for the serial transfer of G-cultures. The mold was accordingly grown in 0.5 per cent peptone water at room temperature. When vegetative growth approached the maximum, the fluid was drawn off from the mycelium and filtered through a sterile Berkefeld W candle. The sterility of the product was tested by adding portions of the fluid to nutrient broth, followed by incubation for one week. Sterility in respect to G-forms was tested by performing a Hauduroy washed-plate series with a portion of the filtrate. No colonies, either of G type or any other type, appeared up to the 8th serial plating. The filtrate was then considered safe to use and was aseptically added to tubes of sterile nutrient broth, in the proportion of 2 cc. filtrate to 6 cc. broth. These tubes were again incubated before use as a check on sterility.

Serial transfer in this medium was then begun, using the GE 17, GC 7, and GS 22 strains which had already undergone 2 months' serial transfer in lithium chloride broth. During 3 months' transfer at the usual 3-day intervals in mold-filtrate medium, all strains showed a slow progressive increase in colony size of the same type which has been recorded as occurring in the lithium-chloride broth series, although the increase occurred more rapidly in the mold-filtrate series. At the end of 3 months a number of still larger colonies appeared on plates bearing mainly the intermediate colony size. When these were replated upon agar, they appeared as typical R colonies of *B. salmonicida*, capable of fermenting glucose, maltose and mannitol, failing to ferment lactose and sucrose, and producing the diffusible brown pigment on agar. Transfer of the newly recovered R strain through 4 passages of 0.1 per cent phenol broth produced numbers

of S type colonies. Cultures grown from these were found again to produce typical "furunculosis" in goldfish.

In procedure (4), 22 G-strains were selected which had been maintained on paraffin wax-sealed agar slants for one year, with transfers only every 3 months. Platings on nutrient agar were made from each strain and incubated for 1 week at room temperature. In 3 instances (strains GE 3, GE 5, GS 12) a few large colonies appeared among numbers of typical G-colonies. These when replated gave rise to the typical R colony form of *B. salmonicida*. Cultures from these colonies gave all the proper cultural and biochemical reactions for the R forms of *B. salmonicida*. Since cultures of this exact description obtained by two other methods had previously been further changed to the pathogenic S form as a final proof of reversion, it was considered that a similar procedure was unnecessary with these strains.

#### SUMMARY

1. Cultures of *Bacillus salmonicida* from infected fish tend to change from the pathogenic S form to a non-pathogenic R form on prolonged cultivation.

2. Dissociation of the R to S type has been shown to occur in single-cell strains of *Bacillus salmonicida* and the R and S dissociants have been described.

3. During the course of dissociative activity in serial lithium chloride broth cultures, a third variant appeared corresponding to the G type culture of Hadley, Delves and Klimek.

4. The same G-variant was also recovered from apparently sterile tubes in the lithium-chloride broth series, by means of the Hauduroy washed-plate technique.

5. Selected G-strains, when subjected to prolonged serial transfer in lithium-chloride broth and in mold-filtrate broth, gave rise to larger colonies, cultures from which proved identical with the known R form of *Bacillus salmonicida*.

6. Dissociation was again enforced upon the R non-pathogenic strains so recovered, resulting in the production of typical S pathogenic strains.

7. Of 22 stock G-strains maintained on nutrient agar, 3 reverted spontaneously to the R form after 12 months.



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# CORRELATED ANTIGENIC AND BIOCHEMICAL PROPERTIES OF STAPHYLOCOCCI

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The general differentiation of pathogenic<sup>1</sup> from non-pathogenic staphylococci on the basis of pigment production and general fermentative activity has been considered by a long series of workers. [Rosenbach (1884), Welch (1891), Gordon (1903 4 and 1904 5), Dudgeon (1908), Winslow and Winslow (1908), Kligler (1913), Winslow, Rothberg and Parsons (1920).] A distinction on an antigenic basis was first made in 1902 by Kolle and Otto (1902) who found that staphylococci from purulent lesions were all agglutinated by immune serums prepared against one of them while strains from other sources were not agglutinated. The majority of the agglutinable strains were orange and were pathogenic for mice. In view of the very large number of confirmatory publications in the fifteen years following Kolle and Otto's paper [Otto (1903), Proescher (1903), Veiel (1904), Kutscher and Konrich (1904), Fraenkel and Bauman (1905), Noguchi (1911), Dreyer and Nothman (1912), Geisse (1913), Walker and Adkinson (1917)] it is curious that workers in more recent years have not been able to differentiate the staphylococci satisfactorily by means of direct agglutination. Hine (1922) found it necessary to use agglutinin absorption but by this technic did prove that

<sup>1</sup> The term "pathogenic" is very loosely applied to staphylococci, usually implying known pathogenicity for human beings. In the present stage of knowledge such an assumption for any particular strain is not warranted. For convenience, however, this term has been used in this paper to indicate strains from human lesions or strains proven to be pathogenic for animals, and the term "non-pathogenic" to indicate strains from other sources or non-pathogenic for animals.

the majority of strains from disease processes were distinct from those from other sources. Julianelle (1922) found no correlation of antigens with source or experimental pathogenicity in his three antigenic groups determined by agglutinin absorption. Hopkins and Barrie (1928) by absorption also established three groups, only one of which was found in disease processes. Dudgeon and Simpson (1927) resorted to a precipitin technic and, although they used only strains from infectious processes, found that pathogenicity for rabbits was correlated with the presence of a specific precipitinogen and, to a less extent, with orange pigment production. Julianelle and Weighard (1935) found that staphylococci from lesions contained a specific precipitable polysaccharide (A) which could be differentiated chemically as well as antigenically from a polysaccharide, (B), found in strains from other sources.

There is considerable disagreement in the literature as to the degree of correlation of hemotoxin production by staphylococci with pathogenicity or antigenicity. This is understandable considering the various technics and types of erythrocytes used. The majority of workers using broth cultures, or their filtrates, with rabbit erythrocytes have reported a considerable, although seldom absolute, correlation. [Neisser and Wechsberg (1901), Otto (1903), van Durme (1903); Veiel (1904), Kutscher and Konrich (1904), Fraenkel and Bauman (1905), Koch (1908), Noguchi (1911).]

Gordon (1903-4) found that the fermentation of mannitol was one property which differentiated staphylococcal strains from lesions, from those from other sources. Dudgeon (1908), Hine (1922), and Dudgeon and Simpson (1927), confirmed and extended his findings.

The ability of certain strains of staphylococci to coagulate oxalated or citrated plasma observed by Loeb (1903) and by Much (1908) was found by Daranyi (1926) to be associated with pathogenicity and with a strong hemotoxic ability. Gross (1931) and Chapman *et al.* (1934) also found a correlation of coagulase production with pathogenicity, hemotoxic activity and orange pigment production.

We are reporting here the results obtained in testing 286 strains of staphylococci isolated from various sources in respect to the various properties discussed above.

### METHODS

The organisms were isolated on rabbits' or sheeps' blood agar plates. Single colonies were picked from two consecutive platings to establish presumptive purity. Inoculations were made into the various test media from 18-hour broth cultures. During the time of the experiments all the strains were preserved in the refrigerator on plain or blood-agar slants. Transfers were made monthly.

#### *Immunization of rabbits*

Rabbits were immunized during a four weeks' period by a series of intravenous injections of saline suspensions of 18-hour agar slant cultures. Heat-killed organisms were given during the first three weeks and living organisms during the final week. The animals were bled ten days after the last injection.

#### *Preparation of antigens*

Flasks containing 50 cc. of meat-infusion broth were inoculated with 18-hour cultures of the organisms and incubated for 4 days. The whole cultures were precipitated over night in the ice box with 4 volumes of 95 per cent alcohol plus 3 drops of glacial acetic acid. The precipitate and organisms were removed by centrifuging and were extracted with 2.5 cc. of N/16 HCl in a water bath at 100°C. for 15 minutes. The mixtures were then neutralized to litmus and centrifuged. The clear supernatants were used as antigens for the precipitin tests.

#### *Precipitin tests*

The precipitin tests were done by means of a capillary technic described previously (Thompson *et al.*, 1936). Equal quantities of serum and antigen were drawn into graded capillary tubes. The tubes were then supported upright by plasticine on microscope slides. Readings of the amount of precipitate were made after 2 hours at room temperature and 18 hours in the refrigerator.

#### *Hemotoxin tests*

The organisms were grown in beef-infusion broth, (pH 6.5) to which was added 0.03 per cent MgSO<sub>4</sub>. The cultures were kept at

37°C. in an atmosphere of 20 per cent CO<sub>2</sub>. After 10 days' growth they were filtered through Mandler filters and various dilutions of the filtrates were titrated for hemotoxin content against equal quantities of 1 per cent washed rabbit erythrocytes. The mixtures were incubated at 37°C. for 1 hour and kept in the refrigerator for 18 hours. Any definite trace of hemolysis was considered as evidence of hemotoxin production.

#### *Mannitol fermentation*

Cultures were inoculated into tubes of 1 per cent Difco mannitol in beef infusion broth. After 3 days' incubation the presence of acid was tested for by adding 2 drops of bromocresol purple.

#### *Coagulase*

Oxalated plasma was obtained by placing 10 cc. of fresh human blood into a centrifuge tube containing 0.02 gram of potassium oxalate, thoroughly mixing, and then centrifuging out the cells. 0.3 cc. of the plasma was mixed with 0.2 cc. of an 18-24 hour broth culture of the organism to be tested. Partial or complete clotting after 2 hours' incubation at 37°C. indicated the production of coagulase.

#### *Pigment*

The pigment was classified as orange, lemon or white by the appearance of the massed growth from a 72-hour agar plate placed upon white filter paper and pressed between two microscopic slides. No attempt was made to classify the various shades between orange and lemon. When any trace of orange color was present the strain was designated as orange.

### RESULTS

Antigens were prepared from all 286 strains and tested for precipitation with serums obtained by immunization against 9 of the strains. In table 1 examples are given of the types of reactions obtained.

Ninety-three strains gave reactions identical with those given by Julianelle's strain 13 and have therefore been designated as group A. Twenty-nine strains were similar to Julianelle's MX and have been designated as group B in conformity with his terminology. Sixty-four strains gave moderate amounts of

precipitate with serum 32 but little or none with the other serums. Since these strains obviously contain an antigen distinct from Julianelle's A and B, this group has been designated as group C. Sixteen strains giving precipitates with both MX and 32 serums have been tentatively designated as group BC. Eighty-six

TABLE I  
*Typical precipitin reactions*

ANTISERUM AGAINST STRAIN NUMBER	AMOUNT OF PRECIPITATE WITH ANTIGEN FROM STRAIN NUMBER														
	13*	6	I	MX*	Co	Cr	Bo	O	32	G	Hn	D	Con	Bsh	Bd
13*	++++	++++	++++	±	±	0	0	0	0	±	±	0	±	0	0
8	++++	++++	++++	0	0	0	0	0	±	0	0	±	0	±	0
I	++++	++++	++++	0	±	±	±	0	0	0	±	0	0	0	0
MX*	±	±	±	++	+	++	±	0	±	++	++	+	0	±	0
32	0	±	0	0	0	±	+	++	++	++	+	++	±	0	0
Designation of group	A			B			C			BC			O		
Number of strains giving each type of reaction	93			29			64			16			86		

\* Strains 13 and MX were kindly sent to us by Dr L. A. Julianelle as typical of his A and B strain respectively.

TABLE 2  
*Biochemical properties of antigenic groups*

ANTI-GENIC GROUP	NUM-BER OF STRAINS	NUMBER OF STRAINS IN EACH ANTIGENIC GROUP SHOWING							
		Orange pigment	Mannitol + Coagulase + Hemotoxin +	Orange pigment	Mannitol fermentation	Coagulase production	Hemotoxin production	Not orange Mannitol neg. Coagulase neg. Hemotoxin neg.	
(A)	93	68 73%	84 90%	75 80%	85 91%	86 92%	2		
(B)	29	1	1	1	1	2	26 89%		
(C)	64	0	2	4	2	2	56 91%		
(BC)	16	0	0	0	0	0	16 100%		
(O)	86	5	18 20%	21 24%	6	13 15%	54 63%		

strains showed no definite precipitate with any serum and have been called group O.

Group A is clearly set apart from the other groups, not only by the specificity of its antigen but also by the much greater amount of precipitate produced.



In table 2 are shown the results of the biochemical tests arranged according to antigenic groups.

The majority of strains of group A gave positive reactions in all or most of the biochemical tests in contrast to the strains of groups B, C, and BC, of which 90 to 100 per cent were entirely negative in all tests.

TABLE 3

*Hemotoxin production on rabbits' blood plates and in CO<sub>2</sub> toxin broth*

ANTIGENIC GROUP	NUMBER OF STRAINS COMPARED	PER CENT SHOWING HEMOLYSIS ON PLATES	PER CENT OF THESE PRODUCING NO HEMOTOXIN IN TOXIN BROTH
A	78	96	2 6
B	29	65	89
C	64	64	95
BC	15	46	100
O	85	61	77

TABLE 4

*Antigenic groupings of strains from different sources*

SOURCES OF CULTURES	NUMBER OF STRAINS	ANTIGENIC GROUP				
		A	B	C	BC	O
Infections	59	46 78%	1	3	1	8 16%
Normal Conjunctiva Nose Throat	191*	41 21%	24 12%	59 29%	15 9%	5+ 28%
Air	24	2	1	4	0	17 73%

\* 178 strains from normal conjunctivae.

A certain degree of correlation with antigenic group was also found for two other properties not included in the table. Forty to sixty per cent of the colonies of groups B, C, and BC strains were definitely viscid but only 9 per cent of group A strains produced viscid colonies. A number of strains in all groups were tested for their power to reduce methylene blue.<sup>2</sup> The speed of

<sup>2</sup> 1 drop of 1 per cent aqueous methylene blue was added to 24-hour broth cultures and the time required for complete reduction at 37°C. was determined.

reduction by the same strains varied from time to time so that no absolute standard could be set. However, when a number of cultures from different groups was tested at the same time, most rapid reduction was always produced by the group A strains.

When comparisons were made of the ability of any of the strains of staphylococci to produce hemolysis of rabbits' blood plates with production of hemotoxin by the method described above, many strains were found which were able to produce marked hemolysis of rabbits' blood plates although no hemotoxin could be detected in the broth filtrates. The results of these comparisons are summarized in table 3.

In table 4 the relationship of antigenic grouping to the sources of the cultures is shown.

Seventy-eight per cent of strains from infectious sources were in antigenic group A; only 8 per cent of these "infection" strains were in groups B, C, and BC.

Fifty per cent of the strains from mucous membranes were members of groups B, C, or BC; 21 per cent were of group A.

Seventy-three per cent of the strains<sup>3</sup> isolated from the air were in group O, giving no precipitates with any of the serums.

#### DISCUSSION

The very clear-cut antigenic differentiation of group A is further supported by the great degree of its correlation with the other biochemical properties and with the origin. This is in harmony with the conception that staphylococci from disease sources tend to fall into a fairly uniform group possessing certain biochemical and antigenic properties differentiating them from "non-pathogenic" staphylococci. In particular it is in agreement with the work of Dudgeon and Simpson (1927) who found that the presence of a specific precipitinogen in staphylococci was correlated with pathogenicity, and with the recent work of Julianelle and

<sup>3</sup> All strains were morphologically Gram-positive cocci in typical irregular clusters on solid medium and produced typical staphylococcus-like colonies. Many did not liquefy gelatin within the three weeks' period allowed.

The majority of the air strains were kindly given to us by Dr. L. Buchbinder of the Bacteriological Division of the New York City Air Pollution Survey.

Weighard (1935) who reported that pathogenic staphylococci possess a specific carbohydrate antigen distinct from a similar carbohydrate present in "non-pathogenic" strains. The antigenic subdivision of the remaining strains is less clear-cut and not correlated with other factors to any great extent so that the groupings are tentative and subject to confirmation. Our results indicate, however, that at least one antigen, in addition to Julianelle's "B" carbohydrate, is present in the staphylococci from mucous membranes, and that these strains may be further subdivided on this basis. This is in agreement with Hine (1922) and with Hopkins and Barrie (1925) both of whom, by agglutinin absorption, divided their "non-pathogenic" strains into two groups.

The majority of the staphylococci from the air may be separated from the "pathogenic" group and from the "mucous membrane" groups in that they possess no acid-soluble, heat-stable antigen in common with these groups.

The discrepancies between the results of the estimation of hemotoxin production in broth cultures and on blood plates are worthy of note. Neisser (1925) described similar differences and suggested that two different mechanisms are concerned.

We believe that the discrepancies are not due to different mechanisms being concerned but to the fact that the 2 per cent blood agar is a more favorable medium for the production of hemotoxin, even without  $\text{CO}_2$ , than is the broth which we used. The hemotoxins produced by the B and C strains on agar plates are soluble; are neutralizable by antitoxin against A strains; and are increased by growth in 20 per cent  $\text{CO}_2$ . All these properties indicate their essential identity with A type toxin.

#### SUMMARY

Heat-stable, acid-soluble antigens prepared from 286 strains of staphylococci from various sources were tested for precipitation with immune serums against 9 of the strains. The organisms were found to fall into 5 groups: Group A, 93 strains including Julianelle's Type A; group B, 29 strains including Julianelle's

Type B; group C, 64 strains; group BC', 16 strains precipitated by both B and C serums; group O, 86 strains not precipitated by any serum.

When the cultures were tested for orange pigment production, mannitol fermentation, coagulase production and hemotoxin production (in broth under 20 per cent  $\text{CO}_2$ ) a marked correlation of these properties with the group A antigen was found. The correlation of hemotoxin production was less marked when hemolysis of rabbits' blood plates was used as a criterion; considerable percentages of groups other than A producing visible hemolysis of the plates. The evidence indicates that the hemotoxins produced by the B and C strains are identical with those produced by the A strains.

A definite correlation of antigenic grouping with the sources of the cultures was evident. Seventy-eight per cent of strains from infections were in group A. Fifty per cent of strains from mucous membranes were in groups B, C', and BC', with 21 per cent and 28 per cent in A and O respectively. Seventy-three per cent of the strains isolated from the air fell into group O.

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# INFLUENCE OF SALTS IN THE DIET ON THE INTESTINAL FLORA OF THE ALBINO RAT<sup>1</sup>

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When the albino rat is restricted to a diet deficient in inorganic salts there invariably occurs a diarrhea (Swanson and Smith, 1932a; 1934), the onset of which may be as early as the third or fourth day, or may be deferred for a week or more. The duration of this condition is usually only two or three days, although it may continue for a longer period. Thereafter, the feces though formed, are somewhat softer than normal and the quantity is low; several days may pass without defecation. There is sometimes, but not always, a recurrence of the diarrhea later in the period of salt restriction.

Intestinal disturbances in animals deprived of inorganic matter have been previously observed. Voit (1880) noted thin, watery feces excreted by dogs during their first 20 days on a low-calcium diet, after which time the feces became normal. Hoffstadt and Johnson (1925) reported definite constipation and lowered aerobic bacterial count in rats on a calcium-deficient diet. Robertson and Doyle (1936) fed a mineral-poor diet to rats and reported intestinal stasis which was relieved by the simultaneous addition of the carbonates of calcium and potassium to the ration.

These irregularities of intestinal function in animals deprived

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of dietary salts suggested a bacteriological study, and the changes observed in the intestinal flora of rats given a low-salt diet in turn prompted an investigation of the changes in the flora produced by the replacement of various salts and groups of salts in the diet.

## EXPERIMENTAL

### *Methods*

*Animals and diets.* Male albino rats from the Connecticut Agricultural Experiment Station strain, weighing  $45 \pm 4$  grams at weaning (21 days old) were allowed free access to an adequate stock ration consisting of modified<sup>3</sup> calf meal (Maynard, 1930) and paste food,<sup>4</sup> supplemented daily with lettuce and yeast. If they attained a weight of  $120 \pm 4$  grams when they were  $34 \pm 2$  days old they were selected for the experiment.

The basal diet used in all the groups except one (group V) consisted per 100 grams of:

	<i>gram</i>
Casein <sup>5</sup> .....	18
Hydrogenated fat <sup>6</sup> ...	27
Dextrin <sup>7</sup> .....	55

Vitamins were provided separately each day by 200 mgm. dried yeast, 5 drops cod-liver oil, 1 ml. of alcoholic extract of wheat germ, and 2 drops of ether extract of wheat germ.

In the basal diet of group V, low-ash lactalbumin<sup>8</sup> replaced the casein; and 160 mgm. of liver extract,<sup>9</sup> 1 ml. of alcoholic extract of wheat germ, and 5 drops of cod-liver oil comprised the vitamin adjuvants.

The appetite of the low-salt rat is poorer than that of the normal rat. Its daily food consumption has been determined in

<sup>3</sup> Modified by the addition of cod liver oil, 3 per cent.

<sup>4</sup> Whole milk powder, 25 per cent; casein, 25 per cent; wheat germ, 20 per cent; lard, 30 per cent.

<sup>5</sup> Described by Swanson and Smith (1932 a).

<sup>6</sup> Crisco.

<sup>7</sup> White, commercial.

<sup>8</sup> Dry basis, total nitrogen, 14.2 per cent; calcium, none; phosphorus, 0.14 per cent. The Dry Milk Company.

<sup>9</sup> Eli Lilly Liver Extract, No. 343.

several previous investigations. In order to eliminate the influence of variations in intake of energy, protein, and vitamins of the diets, all animals in the present study were given each day the amount of food indicated by the average intake of the large number of low-salt rats previously studied, according to the following schedule:

Period	Grams of basal diet given per rat per day
1 to 7 days	7.1
8 to 14 days	6.4
15 to 35 days	5.7
35 to 60 days	5.6

The inorganic supplements given daily were, for the most part, based upon 408 mgm. of Osborne-Mendel (1917) salt mixture, since this amount represents the average quantity consumed daily by normal male rats eating *ad libitum* an adequate synthetic diet containing 4-per-cent of the salt mixture, an amount long considered satisfactory. Formulas were calculated for each diet and for each period in such a manner that the desired amount of inorganic adjuvants was given daily to each rat together with the desired amount of the basal ration. Table I shows the various groups, the mineral supplements, and the amounts of the different elements given daily.

The method insured a fair degree of uniformity in food consumption throughout the groups. As would be expected, some animals did not conform exactly to the schedule. They, when possible, were fed by hand. The animals were given redistilled water, and their caging and care followed the directions of Smith, Cowgill and Croll (1925).

*Bacteriological procedure.* The plate counts of *Lactobacillus acidophilus* and the bacteria other than *L. acidophilus*, mostly of *Escherichia coli*, *Proteus* and *Enterococcus* types, were made at frequent intervals during the feeding period of 60 days or longer. The feces were collected as nearly as possible under aseptic precautions into sterile test tubes. The test tube containing the specimen was then weighed, the feces transferred to a sterile flask with glass beads, and the original container reweighed. The

weight of the specimen was then obtained by difference with accuracy of  $\pm 0.1$  gram. Sterile tap water was added to the fecal sample in the flask to make a dilution of 1:10; the sample was then emulsified by shaking and further dilutions prepared by transferring suitable aliquots into sterile water in dilution bottles.

The dilutions yielding countable plates for the mixed flora (not *L. acidophilus*) were usually 1:10,000 to 1:100,000. The dilu-

TABLE 1  
*Mineral supplement in diets*

GROUP	NUM- BER OF ANI- MALS	MINERAL SUPPLEMENT	MINERAL SUPPLEMENT SUPPLIED DAILY						
			Ca	Mg	Na	K	Cl	P	Total
			mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
I	12	Osborne and Mendel salts*	50.3	7.2	15.4	80.0	21.9	40.9	408
II	10	Osborne and Mendel salts minus sodium chloride potassium†	99.3	7.2	1.7	7.2	3.9	44.5	408
IV	12	Calcium and phosphorus‡	50.3	0.2	1.4	7.0	3.9	40.9	152
V	9	Calcium carbonate, lac- talbumin low-ash	50.3		1.4	5.0		4.0	126
VII	9	Sodium chloride plus potassium§	0.3	0.2	15.4	63.0	24.7	11.6	242
IX	13	Low-salt	0.3	0.2	1.4	7.0	3.9	14.6	
XIII	6	Calcium carbonate	50.3	0.2	1.4	7.0	3.9	14.6	126

\* Prepared as described in Jour. Biol. Chem. **32**, 369, 1917

† Essentially Osborne and Mendel salt mixture with sodium and potassium carbonate and hydrochloric acid omitted; calcium carbonate was increased to 238.1 grams.

‡ Calcium carbonate 404.4 grams; phosphoric acid, 309.6 grams of 85 per cent (186.6 ml. of sp. g. 1.71).

§ Potassium citrate ( $K_2C_6H_5O_7 \cdot H_2O$ ) 207.8 grams; sodium chloride, 35.6 grams.

tions for the *L. acidophilus* were usually 1:1,000,000 to 1:10,000,000. Duplicate plates were occasionally poured for a closer check; however, the plates poured from different dilutions usually served as fairly close checks on each other. In case the flora showed a tendency to decreased or increased counts, suitable adjustments were made in dilutions from which the plates were poured, or a longer series of dilution plates was made. For example, in case of rapidly decreasing *L. acidophilus* counts, plates as low as 1:1000 or 1:10,000 were poured.

For the purpose of plating the mixed flora other than *L. acidophilus* nutrient extract agar (1.5 per cent agar; 1 per cent Bacto peptone; 0.3 per cent Bacto meat extract; 0.5 per cent NaCl; pH 6.8) was used. The incubation time was always 44 to 48 hours at 37°C. Neo-peptone tomato-juice agar was used for plating of *L. acidophilus*. The method of preparation and the composition of this medium is described by Valley and Herter (1935). The plates were incubated for 44 to 48 hours at 37°C., in an atmosphere enriched to 10 per cent with carbon dioxide.

Naturally, in handling large numbers of plates routinely the colonial characteristic of the organism is a deciding factor in obtaining differential counts. The Neo-peptone tomato agar served admirably in this respect. *L. acidophilus* was identified by its typical filamentous, fuzzy (X-type) colony which was easily recognized under hand lens magnification. The Y-type colony was only obtained infrequently. Aside from the colony characteristics, microscopic examinations in stained preparations were also made from time to time. The colonies fished into Neo-peptone tomato broth showed characteristic granular growth and positive fermentation, with acid reaction, of glucose and maltose, while lactose fermentation was doubtful. Many of the freshly isolated strains did not coagulate milk; on repeated transfers, however, the culture may finally coagulate milk in 24 to 48 hours.

It is fully realized that the method of weighing out the original samples "as received" and neglecting to calculate the weight of the sample on an oven-dry basis is open to criticism. Since the moisture content may vary within wide limits the error introduced will theoretically be reflected in the counts obtained. It is further conceded that the errors from this source may be very much more serious in the experiments of short duration in which only a few counts are made or again if undue accuracy is attempted and small variations in plate counts are considered important. However, in the present experiments various counter tendencies were operative, eliminating entirely the errors from this source:

First, the experiments were of sixty days duration or longer, with platings at frequent intervals, to obtain relative numbers.

Second, the results are expressed as averages for a group on a certain diet, hence the daily fluctuation in individual counts, as well as the errors from variable moisture content, were self-compensating. It should be further noticed that fluctuations in successive counts are not considered important unless the changes shown are indications of a permanent tendency or condition.

The responses to a certain dietary treatment were persistent and at times effected nearly a complete disappearance or replacement of one group of organisms by another group.

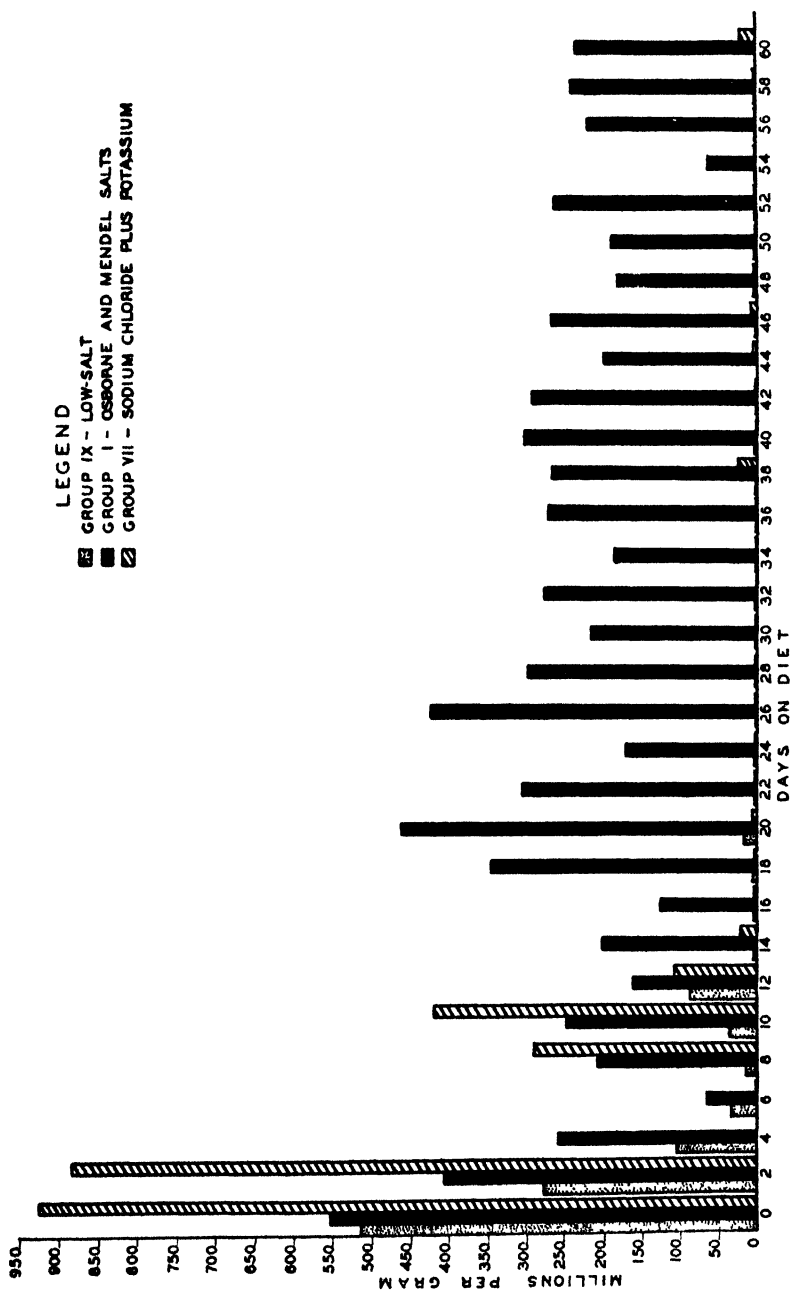
### *Results*

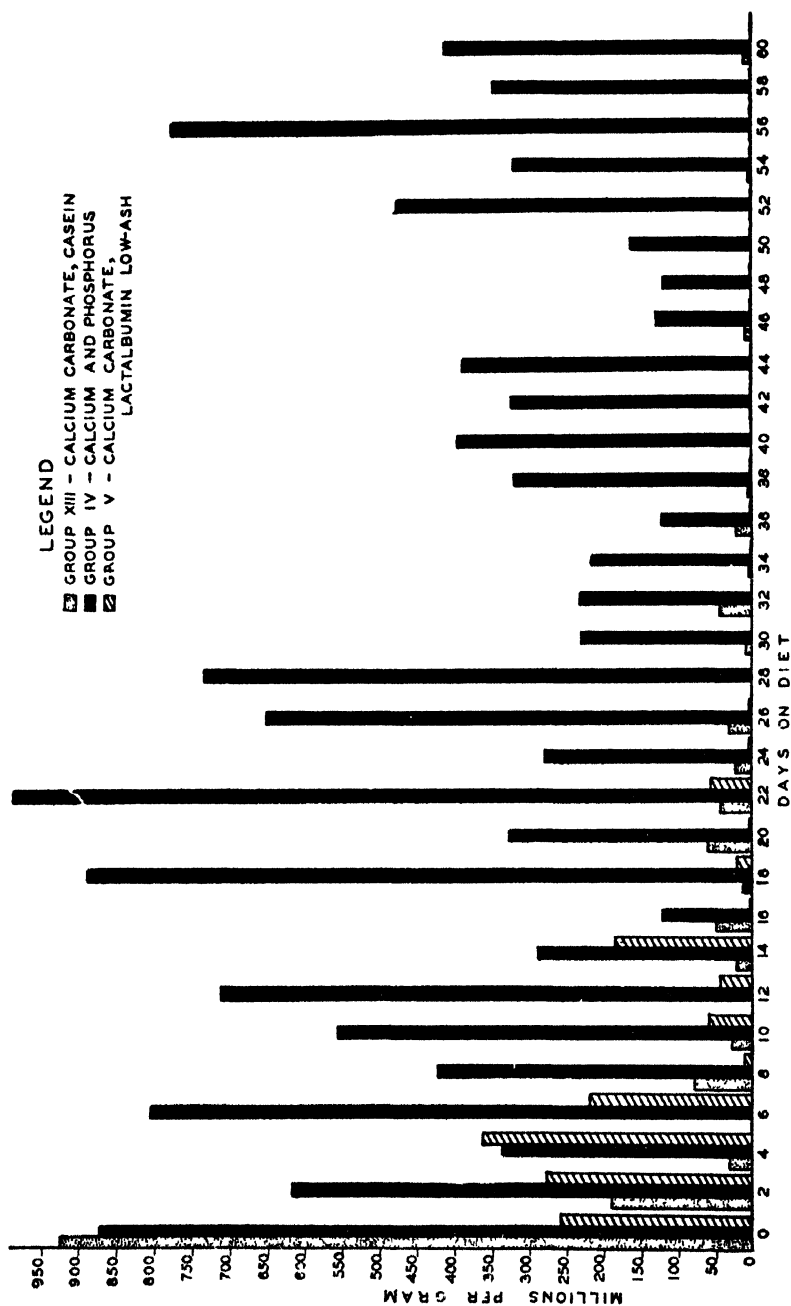
The plate counts per gram of feces both of *L. acidophilus* and of other bacteria made for two consecutive days were averaged for all of the rats of each group. These average counts are shown in charts 1, 2, 3 and 4.

*Group I, Osborne-Mendel salts replaced.* When the rats were transferred from the pre-experimental stock diet to a synthetic ration containing all the salts, no change was observed in the fecal flora. *L. acidophilus* counts of one to three hundred million or more per gram of feces persisted throughout the experimental period of 60 days. Bacteria other than *L. acidophilus* were characteristically scarce, usually not exceeding a few hundred thousand per gram and consisting mainly of organisms of the coli-proteus type, the fecal streptococci and some anaerobes.

*Group IX, low-salt diet.* When the rats were placed on the low-salt diet, *L. acidophilus* without exception gradually disappeared from the intestinal flora, and the bacteria other than *L. acidophilus* increased in number. While the disappearance of the lactobacilli was gradual, the trend was apparent from the beginning of the experimental period. In most cases, after 14 days on the salt-poor diet, the aciduric organisms were largely superseded by other types. In one or two exceptional cases, they persisted in fair numbers for as long as 20 days. In no case did the lactobacilli spontaneously return in appreciable numbers while the rats were on low-salt ration.

Because of the fact that the low-salt rats occasionally failed to eat their food according to the schedule planned and because it

CHART 1. Average counts of *Lactobacillus acidophilus* per gram of feces of albino rat in groups IX, I and VII

CHART 2. Average counts of *Lactobacillus acidophilus* per gram of feces of albino rats in groups XIII, IV and V

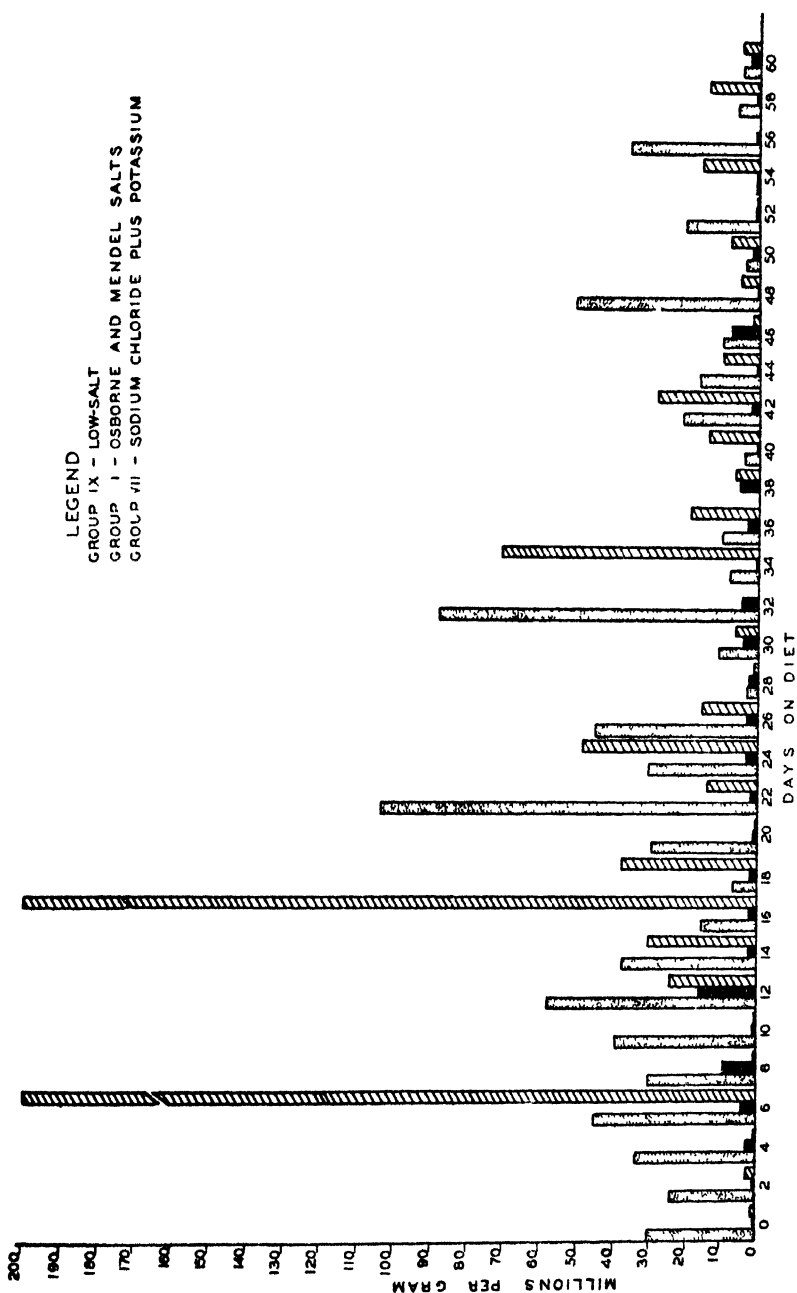


CHART 3. Average c. units of bacteria other than *L. acidophilus* per gram of feces of albino rats in groups IX, I and VII



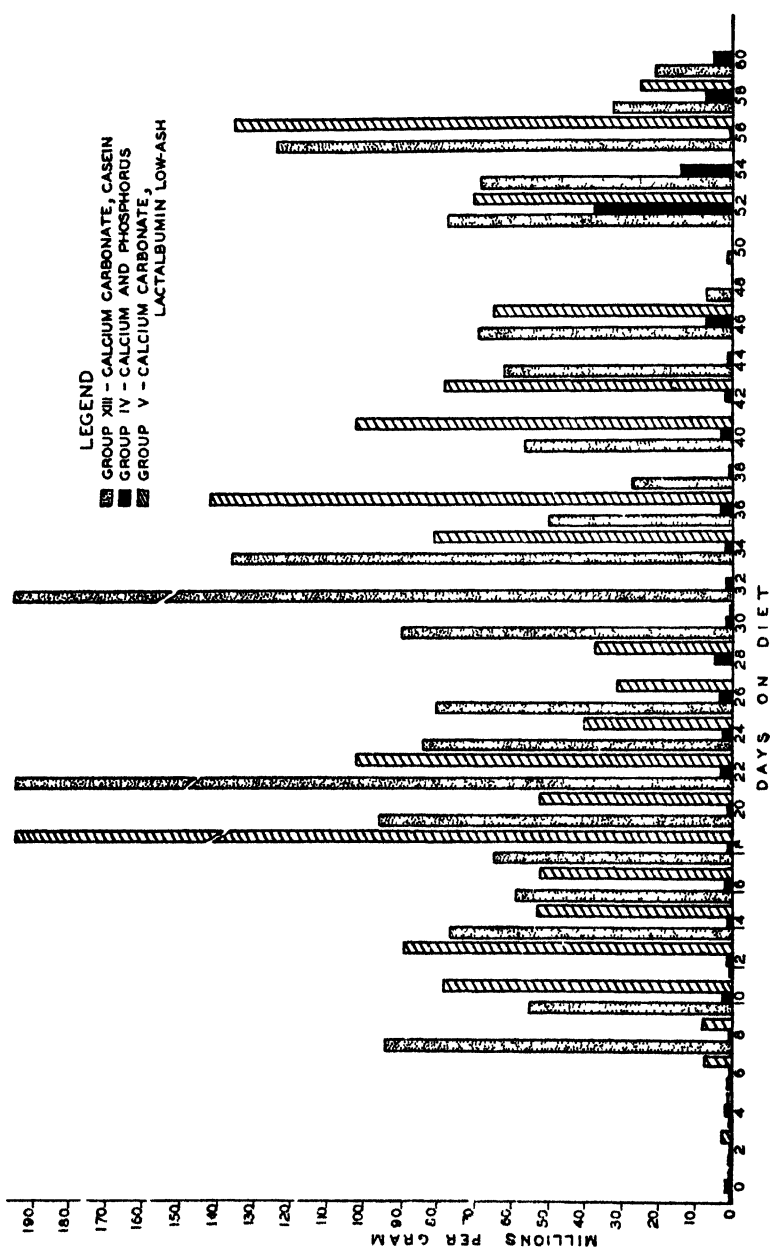


CHART 4. Average counts of bacteria other than *L. acidophilus* per gram of feces of albino rats in groups XIII, IV and V

seemed possible that the disappearance of the lactobacilli might be influenced by and associated with short periods of partial inanition rather than with deprivation of salts alone, four rats were treated strictly according to the paired feeding technique. Confirmatory evidence that salts *per se* influence the normal intestinal flora was thereby secured; the low-salt animals lost their aciduric flora, while those whose diet was supplemented with minerals maintained it in spite of the reduced food intake.

*Group IV, calcium and phosphorus replaced.* Certain ions proved very effective in maintaining the normal flora while others were of no avail (see charts 1 and 2). In group IV, when calcium and phosphorus were replaced in the quantities in which they occur in 408 mgm. of Osborne-Mendel salt mixture, the *L. acidophilus* counts continued very high throughout the period of observation. In fact they were higher on the whole than the counts found for the rats in group I receiving the entire salt mixture. With two of the twelve rats we observed occasional platings which were low in *L. acidophilus*, but the preponderance of the organism in this group was unmistakable. The effect of magnesium was studied to a limited degree. The results obtained with calcium and phosphorus plus magnesium were identical to those obtained with calcium and phosphorus.

Occasional platings were made of the feces of two other groups consuming diets in which calcium and phosphorus were the predominating ions. The results of these corroborated the observations made on group IV. The feces of these groups were exceptionally abundant, yellow in color, and always formed; white streaks, supposedly calcium phosphate, were often apparent.

*Group XIII, calcium carbonate replaced in casein low-salt diet.* Six rats were used in this group.<sup>10</sup> When calcium carbonate alone was added to the casein low-salt diet equivalent in quantity supplied in 408 mgm. of Osborne-Mendel salt mixture, the animals usually ceased to eliminate *L. acidophilus* in feces in much the same manner as did the low-salt rats (see charts 1 and 2), although the aciduric organisms may have prevailed somewhat longer. In

<sup>10</sup> We are indebted to Doctor James M. Orten for the rats in this group. The plan of replacement was identical with that used in the present investigation.

addition, in this group the tendency toward replacement of *L. acidophilus* by *Streptococcus fecalis* was particularly marked. The high counts of bacteria other than *L. acidophilus* were mainly due to the presence of the streptococci.

*Group V, calcium carbonate replaced in lactalbumin low-salt diet.* The intestinal flora of the rats of this group was very similar to the calcium-carbonate-supplemented group just discussed (group XIII). The animals of this group, however, were very different from those of the former. Growth was much more retarded; hyperemia, anorexia, and the usual signs of malnutrition prevailed when the phosphorus in the diet was thus strictly limited.

*Group VII, potassium plus sodium chloride replaced.* The addition of sodium chloride and potassium did not prevent the change in the intestinal flora from the aciduric to the non-aciduric type. That these elements are ineffective in and non-essential to the maintenance of the aciduric intestinal flora was further shown by the continuation of *L. acidophilus* in the fecal matter of rats receiving all of the known dietary essentials except these elements, as in group II. The feces of the potassium- and sodium-supplemented animals were abundant, soft, and in contrast with the calcium-supplemented groups, very black in color.

*Realimentation.* In no case in the low-salt group did the lactobacillus spontaneously return in appreciable numbers; moreover, oral administration of large quantities of the organism failed to reestablish it in the feces of two low-salt rats so treated. However, realimentation with daily doses of 408 mgm. of the Osborne-Mendel salt mixture restored *L. acidophilus* in high counts to the feces of rats which had not shown this organism for 30 to 40 days on the plates poured in as low as 1:1000 dilution. With this quantity of salts in the diet, even though the basal diet (including dextrin) was greatly reduced, it was possible to restore the lactobacilli and to maintain them when as little as three grams of the basal ration were consumed per day.

Inasmuch as the animals given calcium carbonate alone failed to maintain the aciduric flora it seems strange that one rat on the casein low-salt ration when realimented with calcium carbonate regained *L. acidophilus* in its intestinal flora. Further discussion of this point is reserved for another section.

## DISCUSSION

The study of the intestinal flora was undertaken in an effort to explain the diarrhea which occurs when the rapidly-growing albino rat is transferred from the pre-experimental stock diet to the highly purified, synthetic low-salt ration. Because of the favorable influence of dextrin or lactose on the presence of *L. acidophilus* in the intestine (Cheplin and Pettger, 1920) it was anticipated that the change from the stock ration to the diet containing 55 per cent dextrin might be accompanied by a large increase of lactobacilli. The apparent complete disappearance of the organism in certain groups was therefore a surprising phenomenon.

While the intestinal flora of the albino rat has been extensively studied (see Frost and Hankinson, 1931) and considerable information accumulated concerning the fecal bacteria, the influence of the mineral salts on the transformation of the intestinal flora has received little attention. The results of the present study leave little doubt that the inorganic part of the diet influences the intestinal flora, and that even with considerable amounts of favorable carbohydrate (dextrin), the normal flora is not maintained in the absence of certain dietary salts. While it seems true that in animals in poor nutritional state the flora is altered, yet the influences of salts in the diet, as observed in the present study, are direct. The rats of the calcium-carbonate-supplemented group XIII consumed their food regularly; their growth was comparable with that of group I, with Osborne and Mendel salt mixture replaced. The only gross sign of poor nutrition was a slight loss of hair apparent in a few members of the group; yet their intestinal flora was markedly changed. In replacement of ions, it appears that calcium alone is ineffective in maintaining the normal flora. An amount of phosphorus greater than that contained in the casein in the diet is apparently a necessary accompaniment of the calcium. However, phosphorus alone did not prove sufficiently effective to maintain the usual predominance of aciduric flora over the other types, as indicated by the plate counts of the feces of a very limited number of animals receiving sodium phosphate.

The effectiveness of realimentation of low-salt rats with the Osborne-Mendel salt mixture in restoring *L. acidophilus* affords further proof of the specificity of salts in determining the flora of the intestines. With a basal ration as low as 4 grams per day the lactobacilli were restored on the addition of salts and could be maintained in large numbers even on further reduction of the basal diet to 3 grams daily, if at the same time the favorable mixture of salts fed was unchanged. Under such restriction of energy intake the weight of the animal rapidly declined, but the acidophilus count remained high.

From group V (calcium carbonate plus lactalbumin, low-salt) further evidence was secured supporting the theory that both calcium and phosphorus are necessary for the maintenance of the normal flora. The most difficult finding to understand in this series of studies is that calcium carbonate on the one hand was unable to maintain *L. acidophilus* in the calcium-carbonate-supplemented group XIII, whereas on the other hand, in one low-salt rat it was capable of restoring the lactobacilli and of maintaining the aciduric flora over a long period of time. Under this régime the growth of the animal was greatly retarded; the maximum weight attained was 131 grams, which was only a little more than the initial weight. It may be that with growth so restricted, the phosphorus present in the basal ration, together with the added calcium, was sufficient to maintain the flora.

In human subjects the use of acidophilus milk has been found efficacious for the implantation of *L. acidophilus*. Fresh milk and lactose favor the development of acidurics in the intestine as stated by Rettger, Levy, Weinstein and Weiss (1935). The success of implantation of *L. acidophilus* in milk feeding may be due in part to the large amounts of calcium and phosphorus which it contains.

At present it is impossible to suggest a mechanism by which calcium and phosphorus influence the intestinal flora. It is of interest that this property is shared with lactose, which is now thought to play a rôle in calcium metabolism, apparently favoring the absorption and utilization of this element (French and Cowgill; Bergeim, 1926; Kline, Keenan, Elvelijem and Hart,

1932). Phosphorus plays an important part in carbohydrate metabolism and in fermentation of carbohydrate by yeast. It is not inconceivable that it may in a similar manner facilitate the utilization of the carbohydrate by the aciduric organisms. That the mineral content of the diet may alter the H-ion concentration in the intestine and thus influence the existence of *L. acidophilus* is at present problematical, and the evidence concerning this supposition is meager. Recently Friedman (1936) reported that the pH of the feces indicates the type of organisms existing therein. Furthermore, relative to the importance of calcium and phosphorus in the maintenance of the "aciduric" flora, she observed that as rats became rachitic, the number of acid-producing colonies diminished, while the number of non-acid-producing colonies increased. It is apparent however, from her experimental methods and results that *L. acidophilus* was not considered in the study and the acid-producing organisms on brom-cresol-purple lactose agar plates were presumably the lactose fermenters of *Escherichia coli* type.

The present investigation has not shown that *L. acidophilus* accompanied or was the cause of the diarrhea which was typical both of the low-salt group and of all the groups in which calcium was lacking. The occurrence of the diarrhea and the change of the flora were not obviously related; however, it may be significant that scant defecation in some groups was characteristic of the periods during which the lactobacilli were absent. Perhaps, in a majority of cases, the diarrhea preceded the change of flora. Diarrhea in the later stages was frequently accompanied by high counts of coli and proteus type organisms. It appears, however, that, other conditions being kept constant, changes in calcium, phosphorus, potassium, sodium and chlorine induce environmental changes in the intestine which permit certain types of bacteria to proliferate while others are suppressed. The phenomenon may be due to combined physical, chemical, and nutritional conditions (for bacteria) obtaining in the intestine. Whether the effect is directly due to the complete removal of certain essential ions from the intestinal contents thereby rendering the menstruum unsuitable for the growth of certain species or due to the secretion

of products in the intestine bringing about the inhibition is a matter of conjecture. At present we are inclined to emphasize the former view.

### CONCLUSIONS

Lack of salts in the diet is readily reflected in the intestinal flora of the albino rat, the usual aciduric flora with *Lactobacillus acidophilus* predominating being replaced by flora consisting mainly of organisms of the *Escherichia coli* and *Proteus* types and *Streptococcus fecalis*.

The Osborne-Mendel salt mixture in the diet is markedly effective in maintaining a flora high in *Lactobacillus acidophilus*. It also restores the balance of bacillary acidurics in the intestinal flora which has been previously altered by dietary treatment involving deprivation of salts.

Calcium and phosphorus, when added to the low-salt ration, not only prevent the change of flora but sustain the lactobacilli in exceptionally high numbers. Under the experimental conditions of the present study, neither calcium nor phosphorus alone suffices for the maintenance of the normal flora.

*Lactobacillus acidophilus* does not flourish in the intestines of rats when sodium chloride and potassium comprise the inorganic supplement; neither does the withdrawal of these ions from the Osborne-Mendel salt mixture lead to the disappearance of *Lactobacillus acidophilus*.

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# A PRACTICAL CLASSIFICATION OF THE MONILIAS

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The bacteriologist is frequently confronted with the problem of identifying a yeastlike fungus isolated from some part of the human body. Mycelia-producing yeastlike organisms have long been known to play an important rôle in disorders such as thrush, but only recently has etiologic significance been given to these fungi in certain dermatoses and pulmonary diseases. These fungi have also been found frequently in association with certain debilitating diseases such as carcinoma and tuberculosis and have been isolated from individuals with no demonstrable pathologic lesions. It is obvious that little progress will be made in the understanding of the relation of these organisms to disease processes until there is established a practical method for their identification and classification. Since the bacteriologist isolates these fungi from the patient the methods of identification should be adapted to the resources of the bacteriologic laboratory, and the differential criteria upon which identification is based should be easily recognizable.

Benham (1931), Shrewsbury (1934) and Langeron and Talice (1932) have discussed the complicated story of the attempts to place these organisms in their proper botanical position. Berkhout (1923), Ota (1924 a, b), Redaelli and Ciferri (1929), Langeron and Talice (1932) and others have carried out laborious and detailed morphologic studies of this group, but no two authors have agreed on the proper taxonomic position of the fungi con-

<sup>1</sup> Presented before the Society of American Bacteriologists, Indianapolis, Ind., December, 1936.

cerned. It is recognized by all medical mycologists that it is incorrect to use the term *Monilia* as a generic name for these yeastlike organisms because of the prior use of this term as a designation for a wholly unrelated group of fungi. Shrewsbury (1934) has called attention to this fact and proposed that the precise taxonomic position be decided at a meeting of the International Congress of Mycologists. We, however, agree with Benham (1931) that the name *Monilia*, established by usage in the medical literature, should be retained as long as proof of a sexual phase completing the life cycle of the fungus remains undiscovered.

Attempts to classify such a primitive group of organisms as the Monilias on a purely morphologic basis are greatly handicapped by the fact that reproduction occurs only by budding and the formation of mycelial threads. On the other hand a classification based purely on biologic reactions such as the fermentation of various and rare carbohydrates, as advised by Castellani (1919), is not practical. By this method Castellani (1916) isolated seven different species of *Monilia* from eleven cases of thrush in one locality, London. Such a finding suggests that either the method of classification is inaccurate or that there is little hope of correlating the clinical picture with the species of fungus isolated from the lesion. Benham's (1931) careful study of the "medical" *Monilias* represents a logical and careful approach to the problem of classification and she was able to separate these fungi into definite species by correlating macroscopic and microscopic morphology, fermentation reactions and serologic differences. Stovall and Bubolz (1932) compared more than two hundred strains isolated from sputum and recognized three different types by their characteristic colonies on malt agar, their carbohydrate fermentations and their action on milk. The dependence of the carbohydrate fermentation reactions upon the conditions under which the tests are carried out is well illustrated by minor differences in reactions obtained by different observers with fungi which are obviously members of the same species.

In attempting to classify 124 strains of yeastlike fungi isolated

from patients in Duke Hospital we frequently encountered organisms which we were unable to classify by the methods described by Stovall or Benham. A technique was developed, however, which was satisfactory in our hands for classifying the yeastlike fungi isolated from the vaginal tract (Jones and Martin, 1937) and the same procedures were found applicable to the identification of organisms found in other parts of the body. In addition to the 124 strains isolated by us, 29 strains of *Monilia* from various parts of this country and England were compared by our method with 19 representative strains obtained from Benham (1931), Stovall and Bubolz (1932), Langeron and Talice (1932) and Reed and Johnstone (1935).

The method to be described has the advantages of using only simple, easily prepared media and the criteria upon which separation is based are sufficiently distinctive to make identification of these organisms reasonably certain for the bacteriologist without previous mycologic training.

#### SOURCES OF CULTURES

The unknown strains were obtained from the following sources:

	<i>Isolated at Duke Hospital</i>	<i>Isolated elsewhere<sup>1</sup></i>	<i>Total</i>
Sputum.. . . .	47	20	67
Vagina . . . . .	59	0	59
Feces . . . . .	8	0	8
Skin.... .	3	1	4
Lungs (post-mortem) .. .	3	1	4
Liver (post-mortem) .. .	1	0	1
Bile . . . . .	0	1	1
Throat..... .	0	2	2
Tongue. . . . .	1	1	2
Pleural fluid . . . . .	0	1	1
Dog feces . . . . .	2	0	2
Lymph node.. . . .	0	1	1
Source unknown. . . . .	0	1	1
	124	29	153

<sup>1</sup> These strains were obtained through the courtesy of various physicians in this country. Six strains were sent by Dr. Haler of London. The geographical distribution is rather wide: North Carolina 8 strains, New York 4 strains, Massachusetts 3 strains, 2 strains from Arizona, Alabama and Virginia, and 1 strain from New Jersey and Colorado, respectively.

The following 19 "known strains" were studied comparatively by the same methods used for the identification of the cultures listed above:

From Dr. Benham

87 *Monilia albicans* Benham's no. 1755

103 *Monilia albicans* Benham's no. 1773

69 *Monilia Krusei* Benham's no. 1763

88 *Monilia parapsilosis* Benham's no. 1735

114 *Monilia candida* Benham's no. 1956, ATCC no. 2113

From Drs. Stovall and Bubolz

91 *Monilia* type I

92 *Monilia* type II

93 *Monilia* type III

From Drs. Langeron and Talice

46 *Mycotorula psilosis* Their no. 340

44 *Mycotoruloides oralis* Their no. 296

C-70 *Geotrichoides Krusei* Their no. 683

49 *Candida tropicalis* Their no. 255

C-76 *Candida parapsilosis* Their no. 341

171 *Blastodendron intermedium* Their no. 493

47 *Mycocandida northcotei* Their no. 516

From Drs. Reed and Johnstone

238 *Monilia* type II

239 *Monilia* type III

240 *Monilia* type IV

242 *Monilia* type VI

#### METHODS OF IDENTIFICATION

Although the methods proposed in this paper may be found applicable to the identification of other types of yeastlike fungi, only data pertaining to the genus *Monilia* are presented in this paper. We are using the generic name *Monilia* as it has been generally interpreted in the medical literature; i.e., as including yeastlike fungi which reproduce by budding, by the formation of mycelial threads under certain conditions, and which never form asci or aerial hyphae. The 169 identified strains of *Monilia* were divided into 6 species, including the 4 species described by Benham (1931).

No single procedure can be relied upon for identification of all species and all the steps outlined should be performed. Since it has been found that most variations can be accounted for by

slight differences in composition and pH of the various substrata, the methods of making the necessary media are included in the appendix.

An outline of the procedures necessary for identification is as follows:

(1) The fungus is isolated on Sabouraud's glucose agar slant, transplanted to (2) Sabouraud's glucose acid broth and incubated at 37°C. for 48 hours. After noting the type of surface growth, the tube is then shaken to suspend the sedimented organisms and (3) streaked on a beef-extract blood agar plate of pH 7.4, which is incubated at 37°C. for 10 days; the type of colony is noted, and (4) a well-isolated colony is picked and transplanted to a Sabouraud's glucose agar slant. This is incubated at room temperature or 37°C. for 24 or 48 hours. Some of the growth is transplanted to a carrot plug which is kept at room temperature and subsequently examined for asci. The rest of the material is streaked (5) on the surface of a beef-extract agar slant pH 7.4. The growth is subcultured on this medium for 2 or 3 generations and (6) a loopful is streaked on a corn-meal agar slide culture which is incubated at room temperature in a moist sterile chamber for several days. The slide is then fixed, stained and examined microscopically for details of mycelial growth. (7) Four beef-extract broth tubes, containing 1 per cent of glucose, sucrose, lactose and maltose respectively, are inoculated with a pipette containing a saline suspension of the last transplant of the fungus on the beef extract agar slant.

#### GROWTH ON SABOURAUD'S GLUCOSE AGAR AND SABOURAUD'S GLUCOSE BROTH

All of our strains of *Monilia* grew luxuriantly on Sabouraud's glucose agar and this medium has proved entirely satisfactory for the isolation of these fungi from all parts of the body. The organism develops quickly at room temperature or 37°C. and in all species except *M. Krusei* the colonies are raised, white and appear moist and "creamy." Microscopic examination of the unstained organisms shows round or oval yeastlike budding cells with an occasional short strand of mycelium. Langeron and

Talice (1932) have elaborated on the size and shape of these cells (blastospores) and the position of the buds. In our experience the irregularities of these features are so great that no diagnostic significance can be attached to them, with the possible exception of *M. Krusei*, in which the blastospores are long and narrow. Direct microscopic examination, however, should not be omitted since a young colony of *Geotrichum* may resemble a *Monilia*, but can be differentiated easily by the characteristic oblong square-ended spores of the former fungus.

Growth in glucose broth, especially in hanging-drop preparations, has been frequently used to study the mycelial development of the various species. In our scheme of identification, the Sabouraud's glucose acid broth (see appendix) is used only for test tube culture and serves three important purposes: (1) Purification of the culture in instances where bacteria occur as contaminants in the Sabouraud's isolation slants. The acidity and high carbohydrate content of this medium apparently inhibit bacterial growth and favor the development of the fungus. Bacteria, if present, are detected subsequently by plating the broth culture on blood agar. (2) Certain species of *Monilia* present a very characteristic type of surface growth in this medium and (3) this broth culture must be the inoculum of the blood agar plate if characteristic colonies are to be obtained on this medium.

All broth tubes are incubated for 48 hours at 37°C. and examined for surface growth (plate 1). Irregularities may occur if the tubes are incubated for a longer time and the procedure loses diagnostic value. In cultures of *M. Krusei* a dry-appearing surface film is formed which extends up the sides of the tube to a distance of 5 to 6 mm. above the surface of the medium. A similar surface growth occurs with *M. candida* but the film is broken up by numerous small bubbles of gas and the extension of the growth along the sides of the tube is less (2 to 3 mm.). No surface growth occurs in the other 4 species within 48 hours. Characteristic growth may or may not be obtained if bacterial contaminants are present and the procedure should be repeated with a pure culture if bacteria are found on the blood agar plate inoculated from the broth.

## COLONY FORMATION ON BLOOD AGAR

Benham (1931) and Langeron and Talice (1932) have attached diagnostic significance to giant colony characteristics. We attempted a differentiation of species by noting the gross characteristics of 50 strains grown on Sabouraud's honey agar medium for 1 month. Although many of the strains showed differences which might be regarded as characteristic by one very experienced in this type of study, irregularities occurred so frequently that this procedure has been omitted from our scheme of classification.

In the early experiments blood agar plates were streaked from the Sabouraud's broth culture to detect bacterial contamination. The different fungus species, however, grew so differently that the type of colony produced has been used subsequently as a valuable procedure in species differentiation. The plates are streaked from the Sabouraud's broth culture after shaking the tube to resuspend the sedimented organisms. If the plates are streaked from Sabouraud's glucose agar or beef extract agar, colony formation may or may not be characteristic and we believe that the glucose present in the broth inoculum may serve to stimulate growth. All plates are incubated at 37°C. for 10 days. The following descriptions of these colonies present only those features which have proved to be the most constant and of most value in species diagnosis (plate 2).

*Monilia albicans*. Well-isolated colonies are approximately 1.5 mm. in diameter, are more or less circular in outline and have a smooth border. The surface is slightly convex and the whole colony is dull grayish-white in color.

*Monilia parapsilosis*. Well-isolated colonies are smaller than those of *M. albicans* (0.6 to 0.8 mm. in diameter), the outline is smooth and circular and the surface is quite convex. The color is a definite pearly white.

*Monilia candida*. The fungus on blood agar produces large colonies which may attain sizes of 2 mm. and over. The colonies are circular but the most characteristic feature is the mycelial fringe which surrounds the entire colony. These mycelia grow beneath the surface of the medium and form a zone approxi-



mately 1 mm. in width. The color of the colony is grayish-white but is less dull in appearance than the colonies of *M. albicans*.

*Monilia Krusei*. The growth of these organisms is characterized by the great variations in size and shape which occur in colonies which are equally well isolated. The colonies vary from 0.2 to 1.0 mm. in size and the borders may be round and smooth or irregular. The surface may be smooth, ridged, or nodular and heaped up in the center or flat.

*Monilia mortifera*. Only four strains were studied but all of these produced colonies approximately 0.5 mm. in diameter which in general resembled the smaller colonies of *M. Krusei*.

*Monilia stellatoidea*. This fungus produces large colonies which are very characteristic. The colony is composed of a small elevated central zone from which thick tapering "arms" radiate in an irregular manner. These projections are made up of budding cells on the surface of the medium. Between the surface projections mycelial threads can be seen extending beneath the surface of the medium. The colony is readily distinguished from that of *M. candida* by the thick radiating "arms" on the surface which give the whole colony the appearance of a "star in the sky."

Although the colony appearance on blood agar is a distinguishing characteristic of most species, too much importance should not be attached to this feature alone until some experience is acquired in the identification of numerous strains. A single colony on this medium is transplanted to a Sabouraud's glucose agar slant preparatory to subculture on the glucose-free medium. This transplant is necessary to obtain sufficient material to start growth on the beef-extract agar. Carrot plugs (see appendix) are inoculated from the pure culture on the Sabouraud's slant, incubated for several weeks at room temperature and are examined for the presence or absence of large spore-containing cells (asci). Asci could not be found in any of the 172 strains although the cultures were kept for 60 days and examined repeatedly.

#### FERMENTATION REACTIONS

Numerous investigators have attempted to classify these organisms by determining the ability of the fungus to ferment certain

carbohydrates with or without the formation of gas. Stovall and Bubolz (1932) are the only workers who have been able to obtain consistent results with a large number of strains. Cultures of *M. albicans*, *Monilia richmondi*, *Monilia psilosis*, *Monilia Pinoyi*, *Monilia metalondinensis*, *Monilia* (Zillig) and *Monilia pseudotropicalis* obtained from the American Type Collection, showed carbohydrate fermentation identical with their type II (*M. albicans*) when tested by their method although these fungi previously had been classified as to separate species because of their differences in carbohydrate reactions. These authors used brom-thymol-blue as an indicator and read results after incubation at 37°C. for 7 days. Other workers using apparently the same technique have not been as successful in identifying these fungi. Reed and Johnstone (1935) cultured 19 strains of *Monilia* from the feces of patients with various types of gastrointestinal disturbances and attempted to classify them by the method of Stovall and Bubolz (1932) (fermentation of maltose and sucrose, clotting of milk and mycelial growth on malt agar). Six strains were identified as type II (*M. albicans*) and 2 strains as type III (*M. candida*) but the other 11 strains fell into various groups which were designated as types IV, V, and VI. We obtained cultures from these authors and subjected them to the procedures outlined in this paper. Types IV and VI were both identified as *Monilia parapsilosis* (Type I) and type V was identified as a *Cryptococcus*. Hopkins and Hesseltine (1936a) made the interesting observation that variations in carbohydrate fermentation could be obtained by three different observers inoculating the same batch of medium with the same strain of fungus. For example, one observer reported acid and gas in levulose, another obtained acid only and the third observed no acid or gas in this sugar. These authors showed that some of these differences could be accounted for by variations in the size of the inoculum. Benham (1931) also observed that different strains of the same species (*M. albicans*) gave variable results when tested with certain carbohydrates, and Wachowiak and his co-workers (1934) reported that the same strain will vary from time to time, not only losing the power to ferment certain carbo-

hydrates but also apparently being able to acquire this ability. Langeron and Talice (1932) considered the fermentation reactions to be too inconsistent for use as a method of identification. Lamb and Lamb (1935) proposed a method of determining the carbohydrate reactions by testing for the presence or absence of the carbohydrate after incubation for 1 month at 37°C. The results obtained by our method agree in every instance with those of Lamb and Lamb except that sucrose and galactose are not fermented by *M. parapsilosis* when the broth tubes are sealed with vaseline.

Our first observations on the carbohydrate reactions were as irregular as those obtained by most of the previous observers, variations being especially marked with maltose, sucrose, and galactose. The techniques described by Stovall and Bubolz (1932) and Benham (1931) were followed as closely as possible but neither gave constant results in our hands when tested upon a large number of strains. Consistent reactions were finally obtained by developing the technique described by Jones and Martin (1937). Of 169 strains classified into 6 species and tested with 9 carbohydrates, only 3 strains presented exceptions. One of the 96 strains of *M. albicans* failed to produce gas in dextrin, 1 strain of *M. candida* did not form gas in galactose and 1 strain of *M. Krusei* did not ferment glucose or levulose. If only the four carbohydrates necessary for diagnosis (glucose, sucrose, lactose and maltose) be considered, there is only 1 exception among the 169 identified strains.

The following points in technique should be emphasized:

1. The organism must be a subculture of the "pure" strain picked from the streaked blood agar plate.
2. The inoculum must be taken from the second or third subculture on the glucose-free medium.
3. The organisms should be suspended in 1.0 to 2.0 cc. sterile saline and pipetted into the broth tubes. This procedure is made necessary by the small amount of growth on the beef extract agar.
4. The broth tubes must be sealed with vaseline. *M. parapsilosis* regularly produces acid in sucrose and galactose if the tubes

are incubated aerobically but does not form acid from either of these carbohydrates when sealed. The vaseline seal also prevents fading of the indicator after becoming acid. Reversal of color or fading may occur, however, if the gas pressure in the tube forces the seal high enough to displace the cotton plug, allowing air to come in contact with the culture.

5. The beef-extract broth should be accurately titrated to pH 7.2 before adding the indicator and autoclaving (see appendix). An increase of 0.2 pH will inhibit the fermentation of sucrose and galactose by *M. albicans* and neither acid nor gas will be produced in glucose or levulose by *M. parapsilosis*.

6. Breakdown of the carbohydrates should be avoided, preferably by sterilizing the 20-per cent carbohydrate solution by filtration.

7. The carbohydrate broths should be used within 2 weeks after the carbohydrate has been added to avoid slight changes in pH on standing.

All data presented in table 1 are readings made after 10 days incubation at 37°C.

The fermentation reactions of *M. Krusei* and *M. parapsilosis* are identical and these species cannot be differentiated by this procedure, but the flat dry growth on Sabouraud's agar, the surface growth on Sabouraud's glucose broth and the irregular poor growth on blood agar easily distinguish *M. Krusei*. Four strains of *M. parapsilosis* produced only a small bubble of gas and 1 strain of *M. Krusei* failed to ferment any carbohydrate whatsoever.

All of the vaginal strains have been tested for their ability to clot milk and the results agreed entirely with those obtained by Stovall and Bubolz (1932), the new species *M. stellatoidea* being similar to *M. albicans* in this respect. The test, although quite satisfactory, is not necessary for diagnosis and was omitted from the study of the other strains.

#### MYCELIAL GROWTH

Langeron and Talice (1932) classify these mycelia-producing yeastlike fungi by criteria based chiefly on differences in the

TABLE 1

NUM- BER OF STRAINS	CULTURES STUDIED	DLX- TROSE	SACCHA- ROSE	LAC- TOSE	MAL- TOSE	MAN- NITE	LEVU- LOSE	GALAC- TOSE	DEX- TRIN	INULIN
<i>Monilia albicans</i> (96 strains)										
2	<i>Monilia albicans</i> (Benham)	AG	A	—	AG	—	AG	A	AG	—
1	<i>Monilia</i> type II (Stovall)	AG	A	—	AG	—	AG	A	AG	—
1	<i>Monilia</i> type II (Reed and Johnstone)	AG	A	—	AG	—	AG	A	AG	—
1	<i>Mycotorula psilosis</i> (Lang. et Talice)	AG	A	—	AG	—	AG	A	AG	—
1	<i>Mycotorulodes ovalis</i> (Lang. et Talice)	AG	A	—	AG	—	AG	A	AG	—
1	<i>Monilia pinoyi</i> (Castellani)	AG	A	—	AG	—	AG	A	AG	—
58	Strains isolated from sputum...	AG	A	—	AG	—	AG	A	AG	—
19	Strains isolated from the vagina	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from the lungs (P.M.)	AG	A	—	AG	—	AG	A*	AG	—
2	Strains isolated from human feces	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from dog feces	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from the skin	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from the tongue	AG	A	—	AG	—	AG	A	AG	—
1	Strain isolated from bile	AG	A	—	AG	—	AG	A	AG	—
1	Strain isolated from a lymph gland	AG	A	—	AG	—	AG	A	AG	—
<i>Monilia parapsilosis</i> (14 strains)										
1	<i>Monilia parapsilosis</i> (Benham)	AG	—	—	—	—	AG	—	—	—
1	<i>Monilia</i> type I (Stovall)	AG	—	—	—	—	AG	—	—	—
1	<i>Monilia</i> type IV (Reed & Johnstone)	AG	—	—	—	—	AG	—	—	—
1	<i>Monilia</i> type VI (Reed & Johnstone)	AG	—	—	—	—	AG	—	—	—
1	<i>Candida parapsilosis</i> (Castellani)	AG	—	—	—	—	AG	—	—	—
3	Strains isolated from the vagina	AG	—	—	—	—	AG	—	—	—
2	Strains isolated from the throat	AG	—	—	—	—	AG	—	—	—
2	Strains isolated from sputum...	AG	—	—	—	—	AG	—	—	—
2	Strains isolated from skin.	AG	—	—	—	—	AG	—	—	—

*Monilia candida* (13 strains)

1	<i>Monilia candida</i> (Benham) . . . . .	AG	AG	—	AG	—	AG	AG	—
1	<i>Monilia</i> type III (Stovall) . . . . .	AG	AG	—	AG	—	AG	AG	AG
1	<i>Monilia</i> type III (Reed and Johnstone) . . . . .	AG	AG	—	AG	—	AG	AG	AG
1	<i>Candida tropicalis</i> (Lang et Talice) . . . . .	AG	AG	—	AG	—	AG	A	AG
1	<i>Blastodendron intermedium</i> (L. et T.) . . . . .	AG	AG	—	AG	—	AG	AG	AG
4	Strains isolated from the vagina . . . . .	AG	AG	—	AG	—	AG	AG	AG
3	Strains isolated from sputum . . . . .	AG	AG	—	AG	—	AG	AG	AG
1	Strain isolated from feces . . . . .	AG	AG	—	AG	—	AG	AG	AG

*Monilia Krusei* (13 strains)

1	<i>Monilia Krusei</i> (Benham) . . . . .	AG	—	—	—	—	AG	—	—
1	<i>Geotrichodes Krusei</i> (Lang et Talice) . . . . .	AG	—	—	—	—	AG	—	—
4	Strains isolated from the vagina . . . . .	AG	—	—	—	—	AG	—	—
2	Strains isolated from sputum . . . . .	AG	—	—	—	—	AG	—	—
1	Strain isolated from sputum . . . . .	—	—	—	—	—	—	—	—
3	Strains isolated from human feces . . . . .	AG	—	—	—	—	AG	—	—
1	Strain isolated from liver (P.M.) . . . . .	AG	—	—	—	—	AG	—	—

*Monilia mortifera* (4 strains)

1	<i>Mycocandida mortifera</i> (Lang. et Talice) . . . . .	AG	AG	AG	—	—	AG	AG	AG
2	Strains isolated from sputum . . . . .	AG	AG	AG	—	—	AG	AG	AG
1	Strain isolated from human feces . . . . .	AG	AG	AG	—	—	AG	AG	AG

*Monilia stellatoidea* (29 strains)

29	Strains isolated from the vagina . . . . .	AG	—	—	AG	—	AG	—	AG
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## Unclassified (3 strains)

1	Strain isolated from human feces . . . . .	AG	A	—	—	—	AG	AG	A
1	Strain isolated from lung (P.M.) . . . . .	AG	A	—	A	—	A	AG	—
1	Strain isolated from pleural fluid . . . . .	AG	A	—	—	—	AG	A	—

\* One strain did not form acid in this carbohydrate.

type of mycelial growth. In any purely morphologic study the question always arises as to the significance of minor differences in structure. These authors not only separate these fungi into different species but into different genera on the basis of variations in mycelial structure. Two strains of *M. psilosis* from Puerto-Rico were studied by them and classified into two different genera, *Mycotorula* and *Mycotoruloides*. We obtained cultures of type species from these authors and found *Mycotorula psilosis* and *Mycotoruloides ovalis* to be indistinguishable from *M. albicans* by any one of the methods described, including reciprocal agglutinin absorption tests. Their genera *Candida* and *Blastodendron* were both identical with our species of *Monilia candida*.

Benham (1931) describes the mycelial growth of the various species on corn-meal agar and emphasized chlamydospore formation in cultures of *M. albicans*. Stovall and Bubolz (1932) differentiate types I, II and III by noting qualitative differences in the amount of mycelial growth around colonies grown for 48 hours on malt agar. This method has not been found reliable in other laboratories. Reed and Johnstone (1935) obtained no mycelial growth on malt agar plates with types IV and VI (shown by us to be identical with type I) and Hopkins and Hesseltine (1936) noted that variations could be produced by varying the thickness of seeding of the plates.

Mycelial development in corn-meal agar slide cultures was studied in all of the 172 strains described. The slide culture technique was essentially the same as that described by Benham (1931) except that no coverslip was used and the preparations were fixed and stained with lactophenol and cotton-blue (see appendix). The inoculum in every case was taken from the second or third subculture of the fungus on the glucose free medium and the inoculating loop was streaked heavily the length of the slide, cutting the surface of the agar. The cultures were incubated at room temperature and after the mycelia had developed the slides were dried in the air and stained. In spite of all efforts to maintain a constant technique variations in the amount of mycelial growth occurred. *M. albicans* most frequently develops a "tree-like" branching mycelium with swollen

thick walled cells (chlamydospores) on the tips of the mycelial branches but occasionally only a single mycelial "twig" bearing a few chlamydospores can be found in the entire slide. If the mycelium develops well certain "typical" structures may be regularly found and the following descriptions are based on only the most constant and characteristic features presented by each species (plate 3). This procedure cannot be omitted from our identification scheme as this step is necessary to rule out the yeastlike fungi which do not form mycelia (*Cryptococcus* and *Saccharomyces*).

*M. albicans* produces a well developed branching "tree-like" mycelium which develops chlamydospores on the tips of most branches. The ball-like structures described by Benham (1931) and Langeron and Talice (1932) occur occasionally but such clumps of spores are rare in corn-meal agar slide cultures inoculated from sugar-free media.

*M. parapsilosis* produces mycelia with difficulty but such a mycelium, when formed, is fairly well developed and branched. No chlamydospores are found.

*M. candida* forms mycelia very readily, the hyphal elements projecting for a considerable distance from the line of streak. Numerous spores are found scattered throughout the mycelial branches. There are no chlamydospores.

*M. Krusei* forms mycelia which, when well developed, appear as naked threads which branch only at wide intervals. The long narrow spores occur in groups which lie in irregular masses resembling a bundle of "crossed sticks." No chlamydospores are found.

*M. mortifera* produces a branched mycelium very similar to that formed by *M. parapsilosis*.

*M. stellatoidea* produces mycelia and dense ball-like clusters are formed with great regularity, thus resembling the structures previously described by Benham (1931) and Langeron and Talice (1932) as characteristic of *M. albicans*. Two of the 29 strains studied had single chlamydospores.

The most striking discrepancy found in the studies of mycelial development was the failure of *M. albicans* to produce the dense



ball-like clusters previously reported as characteristic for this species. This constant finding in our 96 strains is due, we believe, to the use of the scanty growth of the organism on the sugar-free medium as inoculum. Both strains of *M. albicans* from Benham and *Mycotorula silosis* and *Mycotoruloides ovalis* from Langeron and Talice developed chlamydospores but no large clusters. It should be noted that chlamydospores do not form regularly if the corn meal agar is inoculated from Sabouraud's agar or Sabouraud's broth cultures. It is conceivable that the transfer of small amounts of glucose from the inoculum may cause irregularities similar to those previously noted in the carbohydrate fermentation reactions. *M. stellatoidea* regularly produces large clusters but only rarely is a single chlamydospore found.

#### SEROLOGIC STUDIES

The lack of correlation between the morphology and the biologic reactions of these fungi has led several investigators to study the possibility of using a serologic method for differentiation. Immune rabbit sera have been prepared and tested for agglutinins (Benham, 1934, Almon and Stovall, 1934, Hines, 1924) for precipitins (Stone and Garrod, 1931, Kesten et al., 1930, Lamb and Lamb, 1935) and for complement-fixing antibodies (Stone and Garrod, 1931). A study of the results obtained by these authors indicates that antibodies can be obtained in good titer and are of value in confirming an identification established by other methods. The experience of these investigators has shown that *M. albicans* and *M. candida* cannot be differentiated by either precipitin or agglutination tests even after the sera have been absorbed with the appropriate antigen. Cross agglutination among more unrelated species has also been a problem although most of these difficulties can be overcome by the use of specifically absorbed sera. The close antigenic relationship of some of the members of this genus and the variation in titers obtained with various strains of the same species exclude both agglutination and precipitin tests as procedures of practical value in identifying large numbers of strains. For example, in Ben-

ham's paper, table 4, are listed the agglutination reactions of 30 strains of *M. albicans* obtained from various sources and identified morphologically. Eighteen of these strains (60 per cent) were agglutinated to high titer by the homologous serum only, in 4 instances the same titers were obtained with anti-*albicans* and anti-*Krusei* serum and in 8 strains the titer in anti-*Krusei* serum was only slightly less than the titer observed in anti-*albicans* serum. We have run a number of agglutination tests using

TABLE 2

*Maximum agglutinin titers obtained with the 6 species when tested with 3 antisera*

	TITER	M. ALBICANS - 67 TESTS ON 61 STRAINS	M. CANDIDA 15 TESTS ON 11 STRAINS	M. PARAPSILONIA 16 TESTS ON 11 STRAINS	M. KRUSEI 14 TESTS ON 10 STRAINS	M. STELLATODIA 24 TESTS ON 24 STRAINS	M. MORITZURA 2 TESTS ON 2 STRAINS
Anti- <i>albicans</i> serum	1:640	18	5	0	0	0	0
	1:320	29	8	2	0	0	0
	1:160	18	1	6	2	0	0
	1:80	2	1	2	1	5	0
	Less than 1:80	0	0	6	11	19	2
Anti- <i>parapsilons</i> serum	1:640	10	1	12	0	0	0
	1:320	11	3	4	0	0	0
	1:160	15	5	0	3	1	0
	1:80	13	6	0	5	17	0
	Less than 1:80	18	0	0	6	6	2
Anti- <i>Krusei</i> serum	1:640	0	0	0	2	0	0
	1:320	1	0	0	1	0	0
	1:160	15	2	0	6	0	0
	1:80	14	3	3	5	0	1
	Less than 1:80	37	10	13	0	24	1

various immune rabbit sera, with essentially the same results. Attempts were made to control the reaction by using organisms grown on sugar-free media for the test suspension and by using sera prepared by injection of organisms grown on the same medium. Attempts were also made to find the optimal dilution of the suspension to be used in the test as well as the effects of variations in temperature on the agglutinating reaction. Consistently higher titers were obtained when the antigen suspension was diluted 1:1000 by volume and the reaction was read after one

hour incubation in the 55°C. water bath instead of shaking at room temperature or incubating at 37°C. Such an increase in titer did not lead to greater specificity. Since many strains form a granular suspension in the control tube, determinations of exact titers are frequently difficult. Complement-fixation tests were used for the purpose of obtaining a more definite end point but no increase in specificity was attained.

Table 2 summarizes the results of 139 tests on 119 strains of *Monilia* all of which have been identified by the above-described procedures. The results indicate that the serologic reactions are of value, in that the previously described criteria of separation into species can be correlated to some degree with differences in antigenic structure, but may lead to erroneous diagnoses if relied upon for routine identification.

#### PATHOGENICITY EXPERIMENTS

The pathogenicity of *M. albicans* for rabbits has been well established by Benham (1931) and Stovall and Pessin (1933). The latter authors found *M. candida* (type III) to be pathogenic only in enormous doses and *M. parapsilosis* (type I) to be incapable of causing any demonstrable lesion in rabbits. Six strains of *M. albicans* were inoculated intravenously and all the rabbits died in 4 to 5 days with the typical lesions in the kidney described by the above named authors. Three strains of *M. albicans* caused definite abscesses in the skin 48 hours after intracutaneous inoculation. Three strains of *M. stellatoidea* in equivalent doses (approximately one half the growth on a Sabouraud's slant) did not cause death in any of the rabbits. One animal was killed on the seventh day after inoculation and examined carefully for lesions but none could be found. The 2 surviving rabbits were inoculated with *M. albicans* 2 weeks after the initial dose of *M. stellatoidea* and both animals died within 5 days. Three strains of *M. stellatoidea* produced no abscesses after intracutaneous inoculation. Two of the four strains of *M. mortifera* inoculated intravenously and intracutaneously, showed no evidences of pathogenicity. *M. albicans*, therefore, is the only pathogenic species of this genus, if we accept the

ability to produce lesions in the rabbit as evidence that the fungus possesses pathogenic properties for man.

#### UNIDENTIFIED STRAINS

Three strains of *Monilia* could not be identified with any of the 6 species described above. These organisms differed, not only in carbohydrate fermentation and colony formation on blood agar, but in the type of mycelial growth in corn-meal agar. We have avoided describing them as new species because we feel that at least 4 or 5 similar strains should be studied as a group before significance is attached to the failure of an occasional organism to fall into an empiric laboratory classification.

#### SUMMARY OF DIFFERENTIAL CHARACTERISTICS

The following summary describes only the most important and constant features upon which species differentiation is based. The fermentation reactions of only 4 carbohydrates, glucose, sucrose, lactose and maltose, are considered.

*M. albicans*. Creamy colony on Sabouraud's agar; no surface growth in Sabouraud's broth; medium-sized dull gray colony on blood agar; chlamydospores in corn-meal agar; acid and gas formation in glucose and maltose, acid in sucrose.

*M. parapsilosis*. Creamy colony on Sabouraud's agar; no surface growth in Sabouraud's broth; small, raised, pearly white colonies on blood agar; mycelium forms with difficulty in corn-meal agar; acid and gas formation in glucose.

*M. candida*. Creamy colony on Sabouraud's agar; surface growth on Sabouraud's broth characterized by narrow film broken up with bubbles; mycelial fringe around colonies on blood agar; mycelium forms readily in corn-meal agar; acid and gas produced in glucose, sucrose and maltose.

*M. Krusei*. Flat dry colony on Sabouraud's agar; extensive surface growth on Sabouraud's broth; irregularity in size and shape of colonies on blood agar; mycelium shows few branches with spores arranged like "crossed sticks" in corn-meal agar; acid and gas formation in glucose.

*M. mortifera*. Creamy colony on Sabouraud's agar; no surface

growth in Sabouraud's broth; small colonies on blood agar; poorly developed mycelium in corn-meal agar; acid and gas formation in glucose, sucrose and lactose.

*M. stellatoidea*. Creamy colony on Sabouraud's agar; no surface growth in Sabouraud's broth; "star in the sky" appearance of colonies on blood agar; large ball-like clusters of spores in corn-meal agar; acid and gas formation in glucose and maltose.

#### DISCUSSION

Little progress can be made in the understanding and recognition of fungus diseases until there is developed a practical method of identification of the causative agents. Furthermore, the mycologic flora occurring in normal individuals must be established before too much significance can be attached to the finding of yeastlike organisms in the sputum, vagina, skin or feces of a patient. The burden of routine identification falls on the bacteriologist and the methods of identification, therefore, should be adapted to the resources and training of one trained in bacteriologic technique.

In the scheme of identification proposed in this paper, purely mycologic methods (growth at various temperatures, morphology of the conidia, etc.) have been largely disregarded since even a cursory survey of the literature reveals that there is practically no agreement among mycologists as to terminology, classification or methods of study. In the index of Dodge's (1935) book 103 different species names are listed for the genus *Monilia*. An intensive survey of the literature for the criteria by which these species have been classified has not been attempted, since many species have been renamed and reclassified by investigators who did not study the organisms themselves but relied upon published descriptions. The synonymy presented here (charts 1 and 2) is incomplete but is included for the purpose of illustrating the extremely complicated terminology that results from placing too great emphasis on variously interpreted morphologic details.

We agree with Benham (1931) that the generic name *Monilia* should be retained until a perfect stage on the life cycle of these organisms can be demonstrated. The present concern of the

CLASSIFICATIONS PROPOSED BY VARIOUS AUTHORS		CULTURES STUDIED	
CHART 1			
<p><b>CLASSIFICATION PROPOSED BY THE AUTHORS</b></p> <p><i>Monilia albicans</i> (Robin) Zopf 1890</p>	<p><i>Mycotorula albicans</i></p> <p>*<i>Mycotorula psilosotis</i> <i>Mycotoruloides triadatis</i> *<i>Mycotoruloides ovalis</i> <i>Mycotoruloides Aldoi</i> <i>Mycotoruloides unguis</i></p> <p><i>Mycotoruloides sp.</i></p>	<p>Langeron et Talce</p>	<p><i>Mycotorula albicans</i> Lang. et Talce 1932 <i>Parasaccharomyces Harteri</i> Froilano de Mello et al. 1918 "Oidiomycosis" (Sabouraud's collection) <i>Pityrosporum</i> of Dowling (China) Strains isolated from various sources <i>Monilia psilosotis</i> Ashford 1917 <i>Monilia sp.</i> Brocq-Rousseu et al. 1927 Isolated from a case of rhino-pharyngo-stomatitis <i>Monilia Aldoi</i> Pereira <i>Spicaria unguis</i> Weill and Gaudin 1919 <i>Monilia psilosotis</i> Ashford 1917 <i>Monilia Ashfordi</i> Castellani "Oidiomycosis" (Sabouraud's collection) Strains isolated from various sources <i>Endomyces albicans</i> (ATCC 2076) Strains isolated from various sources <i>Monilia psilosotis</i> Ashford 1917 <i>Monilia albicans</i> from thrush (ATCC 2112) <i>Monilia richmondi</i> Shaw 1926 (ATCC 801) <i>Monilia Pinoyi</i> Castellani 1913 (ATCC 752) <i>Monilia</i> from sprue-Zillig (ATCC 4021) <i>Monilia pseudotropicalis</i> Castellani 1913 (ATCC 413) <i>Monilia melalondincensis</i> Castellani 1919 (ATCC 753) Strains isolated from sputum <i>Monilia macedoniensisoides</i> Castellani 1925 <i>Monilia Pinoyi</i> Castellani 1913 87 strains isolated from human sources</p>
	<p>*<i>Monilia albicans</i></p>	Benham	
	<p>*<i>Monilia type II</i></p>	Stovall	
	<p><i>Monilia albicans</i></p>	Authors	
	<p>*<i>Monilia parapsilosis</i> *<i>Monilia type I</i> <i>Monilia parapsilosis</i></p>	Benham Stovall Authors	<p><i>Monilia parapsilosis</i> Ashford 1928 <i>Monilia parapsilosis</i> from skin Strains isolated from sputum <i>Candida parapsilosis</i> Camargo 1934 9 strains isolated from human sources</p>
<p><i>Monilia stellatoidea</i> Jones and Martin 1937</p>	<p><i>Monilia stellatoidea</i></p>	Authors	<p><i>Monilia stellatoidea</i> Jones and Martin 1937</p>

\* Type species studied by the authors.

CHART 2

CLASSIFICATION PROPOSED BY THE AUTHORS	CLASSIFICATIONS PROPOSED BY VARIOUS AUTHORS	CULTURES STUDIED
	<p>{<i>*Candida tropicalis</i> <i>Candida parapsilosis</i> <i>Candida butantanensis</i> <i>Candida</i> sp. *<i>Blastodendron intermedium</i> <i>Blastodendron erectum</i> <i>Blastodendron Krausi</i> <i>Blastodendron Arzti</i> <i>Blastodendron Fareii</i> <i>Blastodendron Braulli</i> *<i>Monilia candida</i></p> <p>{ Langeron et Tallice</p> <p>{ Benham</p>	<p>{<i>Endomyces tropicalis</i> Castellani 1911 <i>Monilia tropicalis</i> Castellani 1913 <i>Monilia parapsilosis</i> Ashford 1928 <i>Monilia butantanensis</i> Gomes 1924 Strains isolated from various sources <i>Blastodendron intermedium</i> Ciferri and Ashford <i>Endomyces albicans</i> (Sabouraud's collection) <i>Blastodendron Krausi</i> Ota 1924 <i>Blastodendron Arzti</i> Ota 1924 <i>Mycelobasidium Fareii</i> Ota 1925 <i>Enantiolhammus Braulli</i> Pinoy 1911 <i>Monilia candida</i> (Thom and Church 4719.1) <i>Monilia candida</i> Bonorden 1851 (ATCC 2113) <i>Monilia candida</i> Bonorden 1851 (ATCC 1369) *<i>Monilia tropicalis</i> Castellani 1913 (ATCC 750) 10 strains isolated from sputum 10 strains isolated from human sources</p>
<i>Monilia candida</i> Bonorden 1851	<p>{ Stovall</p> <p>{ Authors</p>	<p>{<i>Monilia type III</i> <i>Monilia candida</i></p> <p>{<i>*Geotrichoides Krusei</i> <i>Geotrichoides cutaneus</i> <i>Geotrichoides asteroides</i> <i>Geotrichoides Balzeri</i> *<i>Geotrichoides tumefaciens</i> <i>Geotrichoides vulgaris</i> <i>Geotrichoides kefyri</i> <i>Geotrichoides</i> sp. *<i>Monilia Krusei</i> <i>Monilia Krusei</i></p>
<i>Monilia Krusei</i> Castellani and Chalmers 1913	<p>{ Langeron et Tallice</p> <p>{ Benham Authors</p>	<p>{<i>Monilia Krusei</i> Castellani and Chalmers 1913 (<i>Oidium cutaneum</i> Beurmann et al. 1909 *<i>Mycoderma cutaneum</i> Brumpt 1927 <i>Trichosporium asteroides</i> Ota 1926 *<i>Paradomyces Balzeri</i> Gougerot and Burnier 1912 *<i>Monilia Balzeri</i> Brumpt 1922 <i>Monilia tumefaciens alba</i> Foulerton 1900 *<i>Candida - vulgaris</i> Berkhout 1923 <i>Monilia candida</i> Bonorden 1851 pro parte *<i>Monilia Bonordoni</i> Vuillemin 1911 pro parte <i>Candida kefyri</i> Berkhout Strains isolated from various sources *<i>Monilia Krusei</i> from feces 12 strains isolated from human sources</p>
<i>Monilia mortifera</i> n. comb.	<p>{ Langeron et Tallice</p> <p>{ Authors</p>	<p>{<i>*Mycocandida mortifera</i> <i>Mycocandida onychophila</i> *<i>Mycocandida</i> sp. <i>Monilia mortifera</i></p>

\* Type species studied by the authors.

bacteriologist and clinician is the recognition and treatment of fungus disease in the patient. Increase in our knowledge of these problems cannot be expected until there is a practical method of identification and a terminology that is generally accepted.

#### SUMMARY

One hundred and fifty-three unidentified strains of *Monilia* isolated from various sources were studied and compared with 19 "known" species types obtained from other investigators. One hundred and fifty of these organisms could be classified in one of 6 species. The methods used in classification are comparatively simple and the criteria upon which identification is based are easily recognizable if the technique described is rigidly followed.

#### APPENDIX

*Sabouraud's glucose agar*. Bacto-glucose 40 grams, Fairchild's peptone 10 grams, agar 25 grams, distilled water 1000 cc. Melt in autoclave, filter through cotton, tube and sterilize in autoclave at 15 pounds pressure for 15 minutes. No pH adjustment necessary.

*Sabouraud's glucose acid broth*. Same formula as for Sabouraud's glucose agar except that no agar is added. No filtration or pH adjustment is necessary.

*Beef extract agar*. Difco beef extract 3 grams, NaCl 5 grams, Difco peptone 10 grams, agar 25 grams, distilled water 1000 cc. Melt in autoclave, titrate to pH 7.6, filter through cotton, tube and autoclave at 15 pounds pressure for 15 minutes. Final pH 7.4.

*Beef extract blood agar*. Same basic formula as for beef extract agar with the addition of approximately 10 per cent sterile citrated sheep's blood.

*Carbohydrate broth*. Difco beef extract 3 grams, NaCl 5 grams, Difco peptone 10 grams. Make up to 900 cc. in distilled water. Heat to boiling and titrate exactly to pH 7.2. Add 100 cc. of indicator solution (see below), filter and tube in 10 cc. quantities. Autoclave at 15 pounds pressure for exactly 15 minutes. Add 0.5 cc. of a 20 per cent solution of the carbohydrate sterilized by filtration through a Seitz filter. The broth should not be kept for more than 2 or 3 weeks because slight changes in pH may occur.

*Indicator solution*. Brom-thymol-blue 0.04 gram, distilled water 100 cc. Add a small amount of 1 N NaOH to make the solution alkaline. When indicator is in solution, neutralize with 1 N HCl until the exact neutral point is reached and 1 drop of either acid or alkali will cause a complete change of color.

*Corn meal agar*. 62.5 grams corn meal in 1500 cc. water. Heat to 60°C. for one hour. Filter through paper and make volume up to 1500 cc. Add 19 grams of agar. Arnoldize for one and a quarter hours. Filter through cotton, tube and sterilize. No adjustment of pH necessary.



**Carrot plugs.** Carrots are cut into cylinders with a cork borer and made into slants by a long diagonal cut. A small wad of cotton is put in the bottom of the test tube before inserting the carrot and the tube is autoclaved. Sterile distilled water is added at intervals to prevent drying.

**Fixation and staining of slide cultures.** Dehydrate slides by leaving them in air at room temperature for 36 to 48 hours. Stain for 15 minutes with lacto-phenol cotton-blue (see below). Pour off stain and immerse in 70 per cent alcohol for approximately 10 minutes (till agar is almost completely decolorized). Run the slide through 95 per cent alcohol, acetone, a mixture of equal parts of acetone and xylol and finally xylol. Remove from xylol and mount immediately in neutral balsam.

**Lacto-phenol cotton-blue.** Phenol crystals 20 grams, lactic acid 20 cc., glycerol 40 cc., distilled water 20 cc. Dissolve by gentle heat under hot water tap. Add 1 gram cotton-blue.

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## PLATE 1

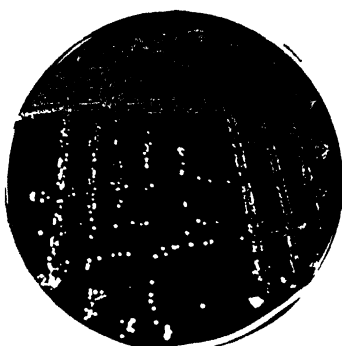
PHOTOGRAPH ILLUSTRATING VARIOUS TYPES OF GROWTH IN SABOURAUD'S  
GLUCOSE ACID BROTH INCUBATED AT 37°C. FOR 48 HOURS

- A. No surface growth (*M. albicans*, *M. parapsilosis*, *M. stellatoidea* or *M. mortifera*).
- B. Bubbly surface growth with thin film (*M. candida*).
- C. Extensive development of surface film (*M. Krusei*).

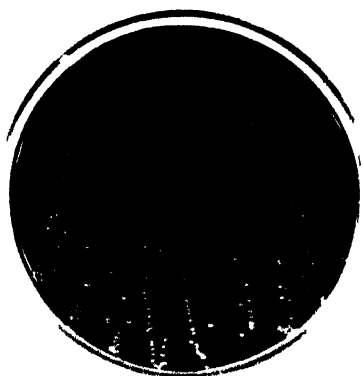


## PLATE 2

PHOTOGRAPHS OF BLOOD AGAR PLATES SHOWING CHARACTERISTIC COLONIES  
AFTER 10 DAYS INCUBATION AT 37 C. FOR 10 DAYS



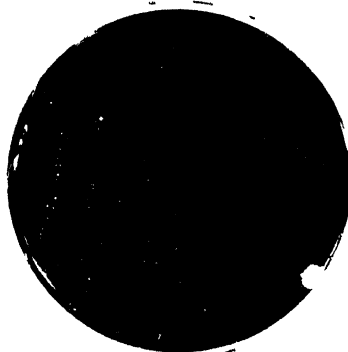
M. PARAPSILOSIS



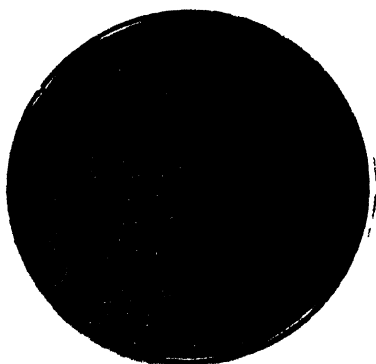
M. ALBICANS



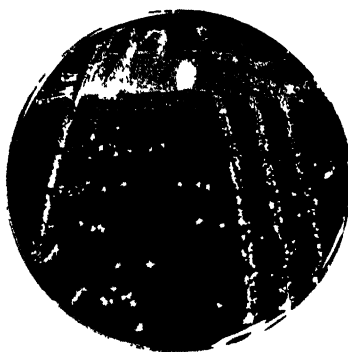
M. CANDIDA



M. KRUSEI



M. MORTIFERA



M. STELLATOIDEA

## PLATE 3

PHOTOMICROGRAPHS ILLUSTRATING TYPES OF MYCELIAL GROWTH IN CORN MEAL  
AGAR AFTER SEVERAL DAYS INCUBATION AT ROOM TEMPERATURE

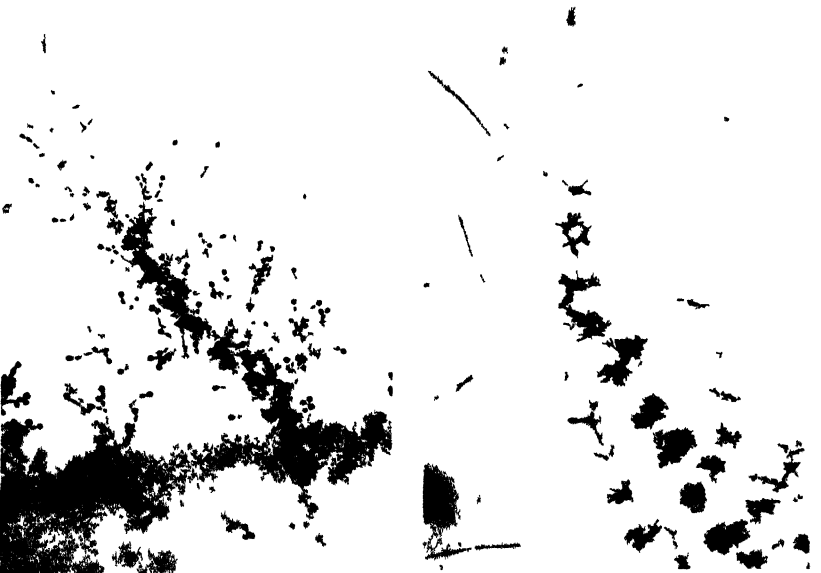


M. PARAPSIOSIS

M. MORTIFERA

M. CANDIDA

M. STELLATOIDEA



M. ALBICANS

M. KRUSEI





# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## EASTERN PENNSYLVANIA CHAPTER (124TH MEETING)

ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH, PRINCETON, N. J., MAY 1, 1937

**BACTERIOPHAGE.** *John H. Northrop*,  
The Rockefeller Institute, Princeton,  
N. J.

Krueger and Northrop found that the kinetics of the action of bacteriophage could be quantitatively accounted for by the following assumptions:

1. The bacteriophage increases in proportion to the percentage increase in bacteria.

2. When a certain critical concentration of bacteriophage to bacteria is reached lysis occurs.

A protein has recently been isolated which possesses the properties of bacteriophage.  $1 \times 10^{-12}$  mgm. of this preparation will cause lysis when added to growing cultures. During the reaction more of the bacteriophage protein is formed. The loss in activity of a solution of the protein is proportional to the denaturation of the protein at various temperatures and at different pH values. The protein is adsorbed by susceptible living or dead bacteria to the same extent as is the bacteriophage activity. Neither the protein nor the bacteriophage activity is adsorbed by resistant bacteria. The rate of diffusion of the protein is the same as that of the active agent and the diffusion coefficient is 0.02 cm.<sup>2</sup>/day, corresponding to a molecular weight of about 500,000.

The protein is not digested nor is the activity decreased by trypsin or papain. Chymo-trypsin inactivates the preparation and this inactivation

is accompanied by the formation of protein insoluble in one quarter saturated ammonium sulfate. There is no detectable hydrolysis of protein during this reaction.

The ultra-violet absorption spectrum agrees with that calculated from Gates' inactivation experiments with ultra-violet light and bacteriophage.

**THE COCCOBACILLIFORM BODIES OF  
FOWL CORYZA AND MOUSE CATARRH.**  
*John B. Nelson*, The Rockefeller Institute, Princeton, N. J.

The isolation of a new type of infective agent associated with catarrhal diseases in chickens and mice, respectively, is reported. Exudate from both hosts contains small Gram-negative cells, referred to as coccobacilliform bodies, which reproduce the essential features of the two diseases on nasal instillation. These bodies are predominantly spherical; generally under  $0.5\mu$  in diameter; filterable through large-pored collodion membranes; and cultivable in tissue cultures. Their growth in tissue cultures is not dependent on living cells but on some diffusible cell constituent. Recently isolated strains show no evidence of growth in nutrient media enriched with blood. The fowl-coryza bodies, however, may survive in blood broth and if carried through an extended series of transfers ultimately grow sparsely in it.

The precise nature of these cocco-

bacilliform bodies is undetermined; their characteristics suggest an intermediate position between the elementary bodies of vaccinia and allied viruses and the bacteria.

**ISOLATION OF MILD STRAINS OF ASTER YELLOW FROM HEAT-TREATED LEAFHOPPERS.** *L. O. Kunkel*, The Rockefeller Institute, Princeton, N. J.

The virus disease, aster yellows, which affects about 170 different kinds of plants and is transmitted by the leafhopper *Cicadula serripata* (Fall), shows great constancy as regards the type of symptoms produced on any one of its

many host species. Strains differing in severity have not been found in nature, but recent experiments have shown that viruliferous leafhoppers held at a temperature of about 32°C. for a few days frequently transmit mild strains instead of typical severe yellows. Twelve strains have been isolated by this method of heat treating the vector. All of the variant strains differ from field yellows in causing a less severe chlorosis of leaves, stems and flowers and less uprightness in habit of growth of infected plants. They also cause less stunting.

### NORTH CENTRAL BRANCH

UNIVERSITY OF WISCONSIN, MAY 7-8, 1937

**PHOSPHORYLATION IN THE LIVING BACTERIAL CELL.** *W. Paul Wiggert and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames, Iowa.

Trichloroacetic acid extracts are obtained from cells of *Aerobacter aerogenes* in the absence of glucose (1) and in the presence of glucose (2). The extracts are analyzed for the various phosphorus esters present. The orthophosphoric acid is less in (2) than in (1) and the difficultly hydrolyzable phosphoric acid is greater in (2) than in (1). The difficultly hydrolyzable phosphorus is computed as (total acid soluble P) - (P determinable after 30 min. hydrolysis in N HCl at 100°C.). Since total acid-soluble P increases in (2) as compared to (1), the difficultly hydrolyzable P has increased, apparently at the expense of both the orthophosphorus and the acid-insoluble phosphorus. The decrease in ortho P and the increase in combined P indicate that phosphorylation is taking place. The cells under the conditions of our experi-

ment have been demonstrated to be at least 75 per cent living by a comparison of plate count and microscopic count. Cells from a 15-day culture of *A. aerogenes*, in which a large majority are dead, brought about no such change as occurred in the experiments described above, so that the changes shown are due to living cells.

**GROWTH-FACTORS FOR THE PROPIONIC AND LACTIC ACID BACTERIA.** *H. G. Wood, A. A. Anderson and C. H. Werkman*, Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa.

Wood, *et al.* (J. Bact. **33**, 227, 1937, Proc. Soc. Exp. Biol. Med. **36**, 217, 1937) and Tatum, *et al.* (Biochem. J. **30**, 1898, 1936) have shown that an ether-soluble acid, vitamin B<sub>1</sub> and lactoflavin stimulate growth of the propionic acid bacteria. In (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> medium *Propionibacterium arabinosum*, 34W, and *Propionibacterium pentosaceum*, 49W, grew vigorously throughout numerous transfers. If any one of the

three stimulants was omitted growth was reduced or prevented. Other propionic cultures 9W, 11W, 23W, 52W, 53W failed to grow on this medium after the third transfer. When hydrolyzed casein or purified amino acids were added, the cultures grew vigorously, but certain of them will grow in the absence of amino acids when additional suitable growth factors (thioglycolic acid and pantothenic acid) are included. There is some evidence of a change in the nutritional requirements of the bacteria through adaptation.

The heterofermentative lactic bacteria require hydrolyzed casein. There are some differences within the group. The ether-soluble acid is essential. Tryptophane is indispensable to some and not to others. With one exception either B<sub>1</sub> or lactoflavin must be present since there is no growth if both are omitted.

NOTES ON THE PHYSIOLOGY OF A D-LACTIC ACID-FORMING ORGANISM. A. A. Anderson, H. G. Wood and C. H. Werkman, Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa.

A description and discussion of the physiology of a lactic-acid-forming organism of the homofermentative type are given. The distinctive characteristics of this organism are: (1) it does not require amino acids or proteins, utilizing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as a nitrogen source in the presence of ether extract of acidified yeast extract plus either vitamin B<sub>1</sub> or lactoflavin, (2) the organism is catalase positive, (3) reduces nitrates, (4) grows well on an agar slant aerobically, but does not grow anaerobically unless a fermentable carbohydrate or suitable hydrogen acceptor is present, (5) produces only dextralactic acid from glucose with a conver-

sion of 97 to 98 per cent, (6) grows vigorously at 55°C. and as high as 60°C. and (7) the carbohydrates fermented, differentiate the organism.

FURTHER STUDIES ON THE FACTORS AFFECTING THE VITAMIN B<sub>1</sub> CONTENT OF YEAST. P. L. Pavcek, W. H. Peterson and C. A. Elvehjem, Departments of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin.

The most important factor in determining the vitamin B<sub>1</sub> content of yeast is the nature of the medium. Bakers' yeast appears to assimilate vitamin B<sub>1</sub> from the medium but will synthesize the vitamin if it is absent.

From grain-wort medium assimilation takes place as readily under anaerobic as under aerobic conditions. From a glucose-salts medium assimilation appears to be better when the medium is aerated.

Synthesis of the vitamin (glucose-salts medium) is promoted by aeration.

Further experiments on the relation of strain of yeast to vitamin B<sub>1</sub> synthesis show that *Endomyces vernalis* possesses unusual ability to synthesize this vitamin. It is possible that the high vitamin B<sub>1</sub> content of this yeast is related to fat metabolism as has been suggested in the case of higher animals.

VARIATIONS IN SALT TOLERANCE OF FACULTATIVE HALOPHILES. G. B. Landerkin and W. C. Frazier, University of Wisconsin.

In a study of salt-tolerant organisms isolated from cheese salt baths it was observed that certain facultative halophiles which had previously been able to develop on both nutrient agar and on the same medium containing fifteen per cent sodium chloride sometimes, at a later transfer, failed to develop on the salt-containing medium. By

transferring several of these cultures directly, and in suspension it was shown experimentally that the amount of inoculum is a determining factor in the initiation of growth on a high salt medium as in all cases excellent growth of the suspension inoculation resulted on the nutrient agar while the salt agar was barren. Only by the direct transfer was growth secured on the high salt medium. It is believed that this factor is of practical significance in enumerating the salt-tolerant organisms by plating on high salt media.

AN EQUATION FOR THE EVALUATION OF THE GERMICIDAL PROPERTIES OF SODIUM HYDROXIDE. *C. R. Arnold, Max Levine and John Sharf, Iowa State College, Ames, Iowa.*

From published data on the germicidal efficiency of sodium hydroxide it was ascertained that the equation relating the killing time, concentration of caustic and temperature may be expressed as

$$\log \Theta = S - a \log C - bt$$

where " $\Theta$ " is the killing time, " $C$ " is the concentration of sodium hydroxide, " $t$ " is the temperature. " $S$ " is a constant characteristic for the test organism, and " $a$ " and " $b$ " are constants characteristic for the germicide.

For sodium hydroxide the equation was found to be

$$\log \Theta = 4.9935 - 1.7943 \log C - 0.05649 t$$

By means of these equations it is possible to ascertain the germicidal equivalents of sodium hydroxide for any designated concentration or temperature or killing time.

INHIBITION AND ABSORPTION OF ANTI-COLI BACTERIOPHAGE. *Philip L.*

*Carpenter, University of Wisconsin, Madison.*

Three strains of bacteriophage which lyse cultures of *Escherichia coli* and *Escherichia-Aerobacter* intermediates were isolated from sewage. These were employed in an investigation of the inhibition of lysis by aqueous bacterial extracts, and absorption of bacteriophage by heat-killed suspensions of coliform bacteria. Tests with 13 strains of coliform bacteria showed that inhibition and absorption are parallel reactions, and it is suggested that the two phenomena are closely related.

By means of inhibition and absorption studies the coliform strains could be divided into five groups. One group inhibited lysis by two bacteriophages, three groups inhibited lysis by a single bacteriophage, while the fifth group exerted no inhibitive action. On the basis of recent work with the *Salmonella* group it may be assumed that these studies have serological significance, and therefore indicate that coliform bacteria possess a complex antigenic structure.

HEAT PENETRATION AND EFFECTIVENESS IN THE CANNING OF MEAT IN THE HOME BY THE PRESSURE COOKER. *C. I. Nelson, North Dakota Agricultural College.*

The data presented in this study were obtained in pursuance of a Bankhead-Jones project and a research project authorized by the W.P.A. Both projects resulted directly from the emergency condition that arose out of the great drought when a large share of the cattle of the state had to be slaughtered to save them from starvation. Meat was canned in 19 F.E.R.A. community canning centers during 1934.

It was apparent then, as it is now, that there is a lack of uniformity in recommended details for canning meat

in tins for domestic use. All emergency canning in North Dakota was done by using tin cans, the necessary sealing device for the cans, and a home-size steam pressure retort.

Experimentation has shown that strict sterility, in the laboratory sense, is probably seldom accomplished. What combination of the variables involved is required to produce a product that will "keep" for a reasonable length of time, will be "safe" for use, and palatable for food was the aim of the experimentation. The chief variables found are steam pressure, the time of processing, the size of the container, the method of cooling, and the type of meat to be canned.

It was demonstrated that

1. Harmless varieties of bacteria exist which survive processing, in a No. 3 can (885 grams of beef), at 15 pounds steam pressure for periods approaching 110 minutes or 65 minutes in a No. 2½ can (725 grams). These organisms can contribute to spoilage, making the meat unfit for use.

2. Toxin-producing anaerobes (*Clostridium botulinum* A and B) were less hardy and did not survive such treatment.

**SPORULATION IN RELATION TO COLONY STRUCTURE, WITH SPECIAL REFERENCE TO *BACILLUS ACETOETHYLICUM*.**  
*H. C. Greene and E. B. Fred, University of Wisconsin.*

**INVESTIGATION OF THE ANTIGENIC RELATIONSHIPS AMONG THE RHIZOBIA OF THE SOYBEAN, COWPEA, AND LUPINE CROSS-INOCULATION GROUPS.**  
*O. A. Bushnell and W. B. Sarles, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin.*

Serological techniques, recognizing the rôles played by flagella and by cap-

sular gums in antigen-antibody reactions, are being applied to the root-nodule bacteria of the soybean, cowpea, and lupine cross-inoculation groups. Anti-sera for 3 types of antigens—the whole untreated cell; the cell less its flagella only; and the cell less both its flagella and its gum—have been prepared from each of 10 strains of root-nodule bacteria representative of the soybean, cowpea and lupine cross-inoculation groups. Although much corroboratory work remains to be done, preliminary agglutination tests employing these antisera suggest that some strains of the organisms from soybean, cowpea, and lupine plants possess common antigens to a considerable degree, while other strains, apparently falling into different serological groups, show no relationships at all.

It is hoped that further work with agglutination, agglutinin-absorption, and precipitation tests will clarify the antigenic relationships among these organisms, and perhaps show some correlation with the frequent inter-crossings which have been observed in cross-inoculation tests with bacteria and plants belonging to these three groups.

**THE INFECTIVE ABILITY OF RHIZOBIA OF THE PEA-VETCH CROSS-INOCULATION GROUP.** *E. W. Ruf and W. B. Sarles, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin.*

The influence of insolation, nitrogen fertilization and source of root-nodule bacteria used as inoculants upon the infective ability of the organisms for seven species of leguminous plants of the pea-vetch cross-inoculation group has been studied in successive greenhouse experiments carried on during a period of seven months. Cultures of *Rhizobium* isolated from species of

*Pisum*, *Vicia*, *Lathyrus*, *Cicer*, *Galactia* and *Phaseolus* were used to inoculate seeds of the Alaska pea, Austrian winter pea, Common vetch, Hairy vetch, Broadbean, Lentil and Chick pea. The plants were grown in sterile, nitrogen-poor sand containing various amounts of ammonium nitrate. No artificial illumination was employed.

Rhizobia isolated from *Phaseolus* and *Galactia* plants failed to form nodules on any of the plant species employed, while the one culture isolated from *Cicer* formed nodules only on the Chick pea.

The different genera, species and varieties of plants of the pea-vetch group varied widely in their susceptibility to infection as indicated by nodule formation. Greater quantities of nitrogen fertilization were needed to inhibit nodule production and cross-inoculation during the early spring months than in the winter.

Great differences were noted in the infective ability of the *Rhizobium leguminosarum* strains used in these experiments, but the infectiveness of any one strain was apparently not governed by its original source, that is, the plant species from which it was isolated.

USE OF DISCRIMINANT FUNCTION FOR DIFFERENTIATING BETWEEN SOILS WITH DIFFERENT AZOTOBACTER POPULATIONS. *William P. Martin and Gertrude M. Cox, Iowa State College.*

GROWTH AND RESPIRATION OF SOME SOIL BACTERIA IN EXPRESSED JUICE OF PLANTS. *D. W. Thorne and P. E. Brown, Iowa State College.*

THE NITROGEN NUTRITION AND GROWTH OF YEAST IN GRAIN WORT. *E. C. Saudek, D. R. Colingsworth, and I. L. Baldwin, University of Wisconsin.*

A study of the changes occurring during the growth of yeast in grain wort revealed that under the conditions employed 75 to 85 per cent of the nitrogen and sugar in the medium were assimilated and destroyed during the first three hours of growth. At the same time the maximum percentage of cell nitrogen and the major part of the yeast crop were produced. During the eight hours of growth the pH of the medium was found to decrease from about 4.0 to 3.0, while the titratable acidity was found first to increase and then decrease until the end of the experiments.

Various samples of malt sprouts were employed in the grain mash. It was found that sprouts differed greatly in their nitrogen content and the amount and types of nitrogen that could be extracted from them. Extractions of relatively high alpha-amino nitrogen content afforded the production of higher yields of yeast.

Methods of supplying the nutrient for yeast growth were investigated. In comparison with the procedure in which all of the nutrient material was mixed before seeding, the gradual addition of the first filtrate (strong wort) to the growing yeast was found to be more beneficial. A more economical utilization of the nutrients and a greater crop of yeast resulted.

AN UNUSUAL OCCURRENCE OF HYDROGENOMONAS SP. *W. W. Umbreit, P. W. Wilson and S. Lee, University of Wisconsin.*

THE "TYROSINASE REACTION" OF THE ACTINOMYCETES. *C. E. Skinner, Department of Bacteriology, University of Minnesota.*

Sixty strains of actinomycetes, freshly isolated from soil, produced a brown to black color on beef peptone

agar and on 15 per cent gelatin in water. All of the more than 60 strains which failed to produce the color in beef peptone agar also failed to produce it in gelatin and water. All of the strains of actinomycetes which produced the black color on beef peptone agar also produced it in four days in Conn's sodium asparaginate-glycerol agar (pH 6.8 and 8.4), to which 0.1 per cent or 0.01 per cent tyrosine was added. None of the strains which failed to produce the black color in beef peptone agar produced it in four days on either of Conn's agar media with tyrosine. Some of the 60 strains which produced the color on beef peptone agar failed to produce the color in Czapek's agar to which tyrosine was added. Czapek's agar media is not favorable for the development of this pigment with all strains of actinomycetes. Some strains which produced the color in beef peptone agar and some which did not, produced a lighter brown color on Czapek's agar without tyrosine and in Conn's media without tyrosine, on longer incubation. This brown pigment produced on longer incubation on nontyrosine media may be distinguished from the darker pigment produced rapidly on media containing tyrosine by its color visually, and by its greater solubility in water, alcohol, and chloroform. Some of the strains which failed to produce any pigment on beef peptone agar produced after longer incubation a hitherto unnoticed red pigment in synthetic media to which tyrosine was added, but not in the same media without tyrosine. This pigment turned gradually to a dark brown on incubation, most rapidly in Conn's pH 8.4 agar. It is concluded that the production of the black color by "chromogenus" actinomycetes is due to tyrosine metabolism. The identity of this pigment and the red

pigment is being investigated. The former is said to be melanin. The latter goes through the color changes of tyrosine when transformed to melanin by enzymatic action.

#### SEASONAL FLUCTUATION OF LAKE BACTERIA IN RELATION TO PLANKTON PRODUCTION. A. T. Henrici, University of Minnesota.

Total net plankton, bacteria per cubic centimeter of water by plate counts, and bacteria per square millimeter per day deposited on glass slides, were determined at weekly intervals throughout the period from melting to freezing of the lake, in a seepage lake during a drought year. There was no appreciable contamination of the lake with material washed in from the surrounding land. Under these conditions, the curves for the bacteria by both methods followed very closely curves for the total plankton, lagging behind about one week. It is concluded, therefore, that the amount of organic matter produced by the plankton determines the growth of bacteria in lake water.

#### INTERMEDIATE METABOLISM OF AEROBACTER. M. Mickelson and C. H. Werkman, Department of Bacteriology, Iowa State College, Ames, Iowa.

Dissimilation of pyruvic acid by the *Escherichia-Aerobacter* group yields acetic and formic acids, carbon dioxide and hydrogen. In addition, *Escherichia coli* and *Citrobacter freundii* reduce pyruvic acid to lactic, whereas *Aerobacter indologenes* forms only acetylmethylcarbinol and 2,3-butyleneglycol as reduction products of pyruvic acid. Pyruvic acid is probably hydrated then split into formic and acetic acids, the former being dehydrogenated into CO<sub>2</sub> and H<sub>2</sub>. *Aerobacter indologenes* reduces acetic acid in the presence



of glucose to acetylmethylcarbinol and 2,3-butyleneglycol; however, no reduction occurs when formic acid replaces glucose as the hydrogen donor. Evidence suggests that a more active form of hydrogen is necessary. Hydrogen from formic acid can, however, reduce acetylmethylcarbinol to 2,3-butyleneglycol. *Escherichia coli* accomplishes this reduction.

*Aerobacter indologenes* forms more oxidized products under aeration. CO<sub>2</sub>, acetic acid and acetylmethylcarbinol are increased while ethyl alcohol is reduced. Aeration under 45 lbs. pressure intensified this behavior.

POSSIBLE FACTORS IN RESISTANCE OF THE OLFACTORY MUCOSA TO ENTRANCE OF POLIOMYELITIS VIRUS. *Paul F. Clark*, University of Wisconsin.

A STUDY OF VARIOUS PROTEIN FRACTIONS FROM OLFACTORY MUCOSA OF THE CALF, WITH ESPECIAL REFERENCE TO RESISTANCE IN POLIOMYELITIS. *Rovelle Allen and Paul F. Clark*, University of Wisconsin.

THE INCIDENCE OF HEMOLYTIC STREPTOCOCCI IN YOUNG ADULTS OF COL-

LEGE AGE. *Arthur W. Frisch, Ray R. Rueckert, and Dorothy L. Chandler*, University of Wisconsin.

BACTERIOLOGIC STUDIES IN A CASE OF ACUTE MENINGITIS DUE TO *BACILLUS INFLUENZAE*. *E. C. Rosenow*, Mayo Clinic.

CULTURAL METHODS FOR THE RECOGNITION OF *C. WELCHII* IN WOUNDS. *Lenore Robinson*, Wisconsin State Laboratory of Hygiene.

THE PRECIPITIN TEST AS A MEANS OF IDENTIFYING ACID-FAST BACTERIA. *Janet McCarter and E. G. Hastings*, University of Wisconsin.

THE INHERITANCE OF FACTORS M AND N IN HUMAN BLOOD. *Francis E. Holford*, University of Wisconsin.

ANTIBODIES FOR *TRICHOMONAS FOETUS*. *Phyllis M. Nelson*, University of Wisconsin.

THE SCHWARTZMANN REACTION APPLIED TO THE STUDY OF CERTAIN FUNGI. *Gordon Worley*, Wisconsin State Laboratory of Hygiene.

# THE PURIFICATION AND CONCENTRATION OF DIPHTHERIA TOXIN

## III. SEPARATION OF TOXIN FROM BACTERIAL PROTEIN

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Diphtheria toxin purified by a variety of methods has always contained bacterial protein. This raises the question whether the predominant protein in purified preparations is a fragment of the bacterial protoplasm which carries the toxin, or is itself the toxin. In a previous paper (Eaton, 1936b) it was shown that in various purified preparations there is no relation between the titer of bacterial precipitinogen and the concentration of toxin. Furthermore, purification apparently reduces considerably the amount of bacterial precipitinogen, relative to toxin.

This paper will describe methods of iso-electric point fractionation used for the separation of toxin and bacterial proteins, and will present evidence that the residuum of bacterial protein left in purified toxin after fractionation constitutes a practically negligible part of the total protein present.

Small amounts of red porphyrin compounds are detectable by spectroscopic examination in purified and concentrated preparations. The possibility that a protein combined with these porphyrins may constitute another bacterial fragment in purified toxins must be considered. From studies of the properties of the porphyrins it appears that they are not firmly bound to any protein in the purified preparations but are strongly adsorbed to protein precipitates. A method will be described for the more complete separation of these colored substances from toxin.

On the basis of fractionation experiments described in the previous paper of this series it was concluded that the purified toxin contained several proteins differing in solubility. It is now evident that some of the protein then believed to be an impurity is actually slightly denatured toxin.

#### METHODS FOR SEPARATING TOXIN FROM BACTERIAL PROTEIN

Three different acid precipitation methods were applied to toxin purified by metal salt precipitation (Method III; Eaton 1936a). The toxin solutions used were quite concentrated, containing 300 to 600 Lf units per cubic centimeter.

1. *Nucleic acid.* An equal volume of 1 per cent nucleic acid solution neutralized with sodium hydroxide is added to the buffered toxin solution. The pH is adjusted to 5.3 with acetic acid and the resulting precipitate is centrifuged down, washed, and dissolved in dilute sodium bicarbonate solution at pH 8.4.

2. *Phosphoric acid at 37°C.* A phosphate buffer solution of the toxin is adjusted to pH 5.0 with phosphoric acid and incubated at 37°C. for one to two hours. A slight denaturation of the toxin is purposely produced in this way in order to lower its solubility. The precipitated toxin is extracted with phosphate buffer at pH 7.0 to remove traces of bacterial protein. Most of the toxin remains undissolved but may be dissolved in very dilute alkali. The pH is then readjusted to 8.4 by adding the requisite quantity of acid sodium phosphate.

3. *Acidified one-third-saturated ammonium sulphate solution.* Ammonium sulphate to one-third of saturation is added to the toxin solution and the pH brought to 6.2. The small amount of colored precipitate is filtered off. The filtrate is then adjusted to pH 5.4 with sulphuric acid and placed in the ice box. The slow formation of a granular precipitate may continue for 48 hours. The precipitate is washed with one-third-saturated ammonium sulphate solution previously adjusted to pH 5.4, and dissolved directly in phosphate buffer. The whole procedure is then repeated.

If the toxin precipitate obtained as just described is suspended in water before adding buffer, denaturation occurs so that most of the toxin becomes insoluble at pH 7.0.

## ISOLATION OF BACTERIAL PROTEIN

Bacterial precipitinogen was detected and measured by means of the ring test using anti-serum obtained by injecting rabbits with washed diphtheria bacilli. Details have been given in the second paper of this series. All tests were read after four hours at room temperature when ring formation is at a maximum.

From the supernatant remaining after the precipitation of the toxin at pH 5.0 to 5.4 the bacterial protein may be salted out by adding ammonium sulphate to 0.8 of saturation. Any toxin remaining is also precipitated. The traces of toxin are separated from the bacterial protein fraction by acidifying the solution to pH 5.0 and incubating overnight. Complete removal of the toxin is indicated by failure of the bacterial protein fraction to bind antitoxin.

After precipitation of the bacterial protein with five per cent trichloroacetic acid, the neutralized supernatant gives no precipitin test but the precipitate redissolved in dilute alkali and neutralized gives a good ring with the antibacterial serum.

## RESULTS OF THE ACID FRACTIONATION PROCEDURES

Fractionation of purified toxin by acid precipitation considerably reduces the titer of bacterial protein against the same antibacterial serum as may be seen from the results presented in table 1. The fourth column of the table gives the highest dilution of the preparation which will form a perceptible ring with the antibacterial serum; the last column, the number of Lf units per cubic centimeter at this titer dilution. A comparison of the Lf values at titer for fractionated and unfractionated toxins indicates that the acid precipitation methods reduce by five- to twenty-fold the amount of bacterial protein per Lf unit. The results of a less complete separation by fractional adsorption on magnesium hydroxide have been tabulated in a previous paper (Eaton, 1936b).

A slightly better separation of toxin and bacterial protein is obtained by one precipitation with nucleic or phosphoric acid than by two successive acid precipitations in the presence of ammonium sulphate. The chief objection to the nucleic acid method is

that it introduces a nitrogenous impurity. The procedure with phosphoric acid produces a denatured toxin showing a considerably increased flocculation time. In concentrated ammonium sulphate solution the toxin is apparently less susceptible to denaturation and more completely precipitated at its iso-electric point. Acid precipitates produced in dilute solutions of electrolyte become partially or completely insoluble at pH 6.0, but toxin precipitated by acid from one-third-saturated ammonium sulphate solu-

TABLE 1

*Relation of bacterial protein titer to Lf values in purified toxin preparations before and after fractionation with acid*

PREPARATION NUMBER	METHOD OF FRACTIONATION	Lf UNITS PER CC.	BACTERIAL PROTEIN TITER	Lf UNITS PER CC. AT TITER
2	Unfractionated	420	1:32	13
2a	Nucleic acid toxin precipitate	360	1:3	120
2b	Supernatant from nucleic acid precipitate	10	1:20	0.5
2c	Phosphoric acid toxin precipitate	135	1:1	135
2d	Supernatant from phosphoric acid precipitate	28	1:16	1.7
1	Unfractionated	500	1:80	6
1a	Acid ammonium sulphate toxin precipitate	500	1:8	62
1b	Same as 1a	500	1:4	125
1c	Bacterial protein fraction*	0	1:100	0
3	Unfractionated	800	1:80	10
3a	Acid ammonium sulphate toxin precipitate	360	1:8	45
3b	Bacterial protein fraction*	0	1:150	0

\* Prepared from supernatant of toxin precipitate as described in the section entitled "Isolation of Bacterial Protein."

tion redissolves readily and completely in phosphate buffer at pH 6.0.

#### ESTIMATION OF THE AMOUNT OF BACTERIAL PROTEIN IN PURIFIED TOXIN

When the actual concentration at titer of a single bacterial precipitinogen is determinable, it is possible to estimate by means of the precipitin test, under certain conditions, the amount of

this precipitinogen in purified toxin preparations. Since the bacterial precipitinogen and the toxin are apparently both proteins containing approximately the same percentage of nitrogen, the calculations may be based on measurements of the protein nitrogen.

The results of these measurements are presented in table 2. The Lf values and bacterial protein titers of these preparations have already been given by the corresponding numbers in table 1. The protein nitrogen values given in the third column of table 2

TABLE 2

*Milligrams of protein nitrogen per cubic centimeter in relation to Lf units of toxin and titer of bacterial protein*

PREPARATION NUMBER*	FRACTION	PROTEIN N	PROTEIN N PER Lf UNIT	PROTEIN N AT TITER OF PRECIPITINOGEN (N/d)	RATIO BACTERIAL TO TOTAL PROTEIN (P <sub>b</sub> /P <sub>t</sub> )
		mgm. per cc.	mgm.	mgm. per cc.	
1	Unfractionated toxin	0 230	0 00046	0 0029	1/6
1a	Toxin	0 263	0 00053	0 033	1/60
1b	Toxin	0 250	0 0005	0 062	1/135
1c	Bacterial protein	0.046	>0 01	0 00046	1/1
3	Unfractionated toxin	0 336	0 00042	0 0042	1/9
3a	Toxin	0.180	0.0005	0 023	1/50
3b	Bacterial protein	0 073	>0 01	0 00049	1/1
2	Unfractionated toxin	0.190	0 00045	0 0075	1/15
2c	Toxin	0.095	0 00063	0 095	1/200

\* Correspond to preparation numbers in table 1.

represent the nitrogen in precipitates produced by five-per-cent trichloroacetic acid from known volumes of solution. The protein-nitrogen-Lf ratios in the fourth column indicate the purity of the toxin and will be discussed later. In the expression N/d, (N) represents the milligrams of protein nitrogen per cubic centimeter and (d) the precipitin-titer dilution of the various preparations tested against the same anti-bacterial serum.

If we assume that the dilution of the purified toxin preparation which just gives a perceptible ring with antibacterial serum contains the same concentration of bacterial precipitinogen as the

dilution at titer of the bacterial protein alone, then the ratio of bacterial protein to total protein in the toxin preparations may be stated thus:

$$\frac{P_b}{P_t} = \frac{N_b/d}{N_t/d}$$

In this equation,

$P_b$  is the concentration of bacterial protein in purified toxin.

$P_t$  is the concentration of total protein in purified toxin.

$N_b/d$  is the concentration of bacterial protein nitrogen at titer in preparations 1c or 3b (bacterial protein).

$N_t/d$  is the concentration of total protein nitrogen at titer (toxin plus bacterial protein) in the purified toxin preparations.

It is obvious from the figures in the fifth column of table 2 that the toxin fractions contain much more protein nitrogen per cubic centimeter at titer than do the corresponding bacterial protein fractions. From the ratio  $P_b/P_t$  in the last column it appears that, in purified toxins before fractionation, bacterial protein represents 1 part in 6 to 1 part in 15 of all the protein. After fractionation the bacterial protein constitutes about 1 part in 50 to 200 or 0.5 per cent to 2 per cent of the total protein.

The error in the precipitin test is admittedly very large; but where not over 10 to 20 per cent of the total protein is bacterial precipitinogen,  $P_b$  is small in relation to  $P_t$  and the error in percentage is correspondingly small.

In crude toxin the protein nitrogen per Lf unit ( $N/Lf$ ) has been found to be 0.0012 mgm. to 0.0035 mgm. If pure toxin contains about 0.0005 mgm. protein nitrogen per Lf unit then in crude toxin about 60 to 90 per cent of the protein nitrogen is in bacterial protein.

#### SEROLOGIC AND CHEMICAL IDENTIFICATION OF BACTERIAL PRECIPITINOGENS IN DIPHTHERIA TOXIN PREPARATIONS

Crude toxin contains several substances which give a precipitin reaction with antibacterial serum. These are in part complex carbohydrates (Hazen 1930). There are also present at least two

precipitinogens which appear to be proteins. These are characterized as follows.

Precipitinogen I is adsorbed from crude toxin solutions on calcium phosphate and is salted out by ammonium sulphate at one-third saturation.

Precipitinogen II is removed from solution by all of the reagents which have been used to precipitate or adsorb toxin. Like toxin it is salted out by ammonium sulphate at 0.4 to 0.7 saturation, but it differs from toxin in remaining in solution at pH 5.0 to 5.4.

Precipitinogen I may be almost completely separated from toxin in the first steps of purification. However, some of precipi-

TABLE 3

*Precipitin tests with undiluted crude, purified, and acid fractionated toxins to demonstrate the identity of the precipitinogen*

PREPARATION	Lf UNITS PER CC.	REACTION WITH UN- TREATED ANTI- BACTERIAL SERUM	REACTION WITH ANTI-BACTERIAL SERUM AB- SORBED WITH PRECIPITINO- GEN II.
Crude toxin . . . . .	20	+++	+
No. 1 unfractionated . . . . .	500	+++++	+
No. 1a fractionated. . . . .	500	+++	—
No. 3 unfractionated . . . . .	800	+++++	—
No. 3a fractionated . . . . .	360	+++	—

tinogen II is carried along with the toxin no matter what purification procedure is used. This precipitinogen may be most completely separated from toxin by acid fractionation.

The possibility that precipitinogens other than II are present in purified toxin must be considered. In order to determine this, tests were done with antibacterial serum treated to remove precipitins for II. The antibacterial serum was mixed with the bacterial protein preparation number 3b (table 1), incubated over night, and the resulting precipitate centrifuged down. The supernatant gave no precipitin test with the isolated bacterial protein which has been designated precipitinogen II.

The results of precipitin tests with serum treated in this way are presented in table 3. Corresponding tests with untreated serum



are included for comparison. As expected, crude toxin gives a precipitin reaction with the absorbed antiserum due to the presence of precipitinogen I and probably other bacterial substances. One of the purified toxins before acid fractionation apparently contained traces of precipitinogen I so that it also gave a positive reaction.

It is evident that the other purified preparations in table 3 contain no detectable bacterial protein other than the one used to saturate the antiserum. This excludes the possibility that the precipitin tests obtained with the acid fractionated toxins are due to another bacterial protein which precipitates only at low dilutions with the antibacterial serum.

#### PROPERTIES OF PURIFIED TOXIN AFTER SEPARATION OF BACTERIAL PROTEIN

The toxic protein left after removal of most of the bacterial protein does not differ materially in its chemical properties from the purified toxin previously described. (Eaton, 1936b) The fractionation with acid removes traces of proteose and peptone so that practically 100 per cent of the nitrogen is precipitated by trichloroacetic acid as protein:

It will be noted from the figures in table 2 that the protein-N/Lf ratios are slightly higher after acid precipitation than before. This is probably due to a slight alteration of part of the toxin by acid. In the preparation precipitated by phosphoric acid at 35°C. (preparation 2c in table 2) the protein-N/Lf ratio increased by about 35 per cent although some of the protein nitrogen had been removed as bacterial protein. The least increase in protein-N/Lf ratios occurred when the acid precipitation was done in one-third-saturated ammonium sulphate solution as with preparations 1a and 3a.

The acid-precipitated preparations show a slight to marked increase in the flocculation time. Acid precipitation may reduce the toxicity by as much as 50 per cent but the acid fractionated toxins still have a toxicity of the order of 0.00005 mgm. of nitrogen per M.L.D. or about one hundred times the toxicity of crude toxin.

Measurements of the specific optical rotation of toxins purified by metal-salt precipitation gave values for  $\alpha_D^{25}$  of  $-45^\circ$  to  $-47^\circ$ . The preparation 1a obtained by acid fractionation showed a specific rotation of  $-43^\circ$ . The difference is within the limits of error in the measurements. Since the additional purification had little effect on the optical rotation the values given probably represent the approximate optical activity of the toxin.

#### PROPERTIES OF SLIGHTLY DENATURED TOXIN

Diphtheria toxin as a native protein is probably not precipitated in the presence of low concentrations of electrolyte at any pH. However, various toxin preparations contain different proportions of protein precipitable by acid under constant conditions. Since toxin denatured to various degrees will become precipitable at any pH up to 7.0, or even above, it is now evident that the acid-precipitable protein is denatured toxin and not a protein impurity as was formerly believed.

It has also been found that a sample of toxin which precipitates between 0.4 and 0.7 saturated ammonium sulphate becomes precipitable at 0.3 saturation after slight denaturation. These alterations in solubility are accompanied by an increased adsorbability on colloidal magnesium or aluminum hydroxide. Thus, a mixture of native and slightly denatured toxin may appear, from experiments on fractional adsorption or fractional precipitation with acid or ammonium sulphate, to consist of two or more proteins. The denatured toxin is the more easily mistaken for an impurity because slight denaturation will increase the N/Lf ratio by as much as 30 per cent.<sup>1</sup>

Since denaturation causes an increased flocculation time it is possible to obtain some indication of whether a certain preparation contains denatured toxin or a mixture of native toxin and an impurity by measuring the Kf. The relation of Kf to denaturation of toxin has been discussed in another paper (Eaton 1936c).

Probably even fresh crude toxin contains some denatured toxin. The fraction precipitated from crude toxin at one-third saturation

<sup>1</sup> For a concise statement of the chemical changes in denaturation of proteins see Mirsky and Pauling (1936).

with ammonium sulphate shows an increased flocculation time which may be due to the denatured state of the toxin in this fraction, not, as stated in a previous publication, to the effect of admixed bacterial protein on the flocculation reaction.

#### PROPERTIES OF THE PORPHYRIN COMPOUNDS IN DIPHTHERIA TOXIN

The red porphyrin compounds described by Coulter and Stone (1931), which are present in crude toxin, may be mostly separated from the purified preparations by adsorption on charcoal or magnesium hydroxide. But traces difficult to remove are detectable in concentrated solutions of purified toxin. The close association of the porphyrins with the protein fractions indicated that these colored substances might be present as protein compounds analogous to hemoglobin.

Purified toxin contains two distinct substances showing absorption spectra. One of these, giving absorption bands with centers at 574 and 537 millimicra, corresponds to the complex porphyrin described by Coulter. The other with bands at 563 and 528 millimicra, in dilute phosphate buffer at pH 7.0, may be the copper compound of coproporphyrin said by Coulter to be a decomposition product of the complex porphyrin. These two substances are present in varying proportions and amounts in different toxin preparations.

The two porphyrins are apparently similar in their chemical properties. Both become extractable by ether from the toxin solutions upon bringing the pH to 5.6. Coulter and Stone extracted their complex porphyrin with ether from crude toxin acidified with acetic acid; probably at a pH near 4.0. The porphyrins will pass from the ether solution to an aqueous solution of sodium phosphate at pH 6.0. Both porphyrins are precipitated from aqueous solutions free of protein near pH 5.6. Coincident with the precipitation a shift of the absorption bands toward the red end of the spectrum occurs. On raising the pH to 6.0 or above the porphyrins redissolve and the absorption bands shift back to their original positions. This behavior indicates that the porphyrins are weak acids insoluble in water but forming

soluble sodium salts above pH 5.8 to 6.0. The fact that the porphyrins are precipitated near the iso-electric point of diphtheria toxin explains the difficulty in separating them from toxin by the purification methods used.

The porphyrins are strongly adsorbed to protein precipitates. The first small precipitate containing denatured toxin which is produced by one-third-saturated ammonium sulphate carries down the greater part of the porphyrin from the toxin solution. Because of this the ammonium sulphate and acid fractionation procedure not only serves to separate out bacterial protein but also frees the toxin of all but very small traces of porphyrin. The porphyrin with absorption bands at 563 and 528 is the most difficult to separate completely from the toxin.

#### DISCUSSION

The bacterial precipitinogen remaining in purified diphtheria toxin after acid fractionation is apparently identical with the bulk of the bacterial protein isolated by fractionation from the purified preparations. This bacterial protein gives a ring with the antiserum used at dilutions of 1:300,000 in terms of grams dry weight. The antibacterial serum, although reactive with very small amounts of bacterial protein, apparently contains no precipitin for about 99 per cent of the protein in the purified toxin preparations. If the toxic protein is derived from the bacterial cells it must be, (1) a substance that is readily soluble in normal saline and hence easily removed by washing the diphtheria bacilli used for production of the antiserum; or (2) a protein that does not produce precipitating antibodies in rabbits. Diphtheria toxin is a substance which dissolves out of the bacterial cells into the medium; and it has been reported that antitoxin produced in rabbits will not flocculate with the toxin. The Wadsworth-Wheeler medium used for the production of toxin contains no protein when sterile so that the protein under consideration could not have come from the culture medium.

These considerations do not exclude the possibility that a readily-soluble protein other than toxin is elaborated by the diphtheria bacillus. However, the close relationship between dena-

turation of the toxic protein and alterations in its reactivity with antitoxin, and the correlation of Lf values, toxicity, and protein nitrogen in the purified preparations make this explanation seem improbable. If the protein were merely a carrier of the toxin the relative amounts of protein, flocculating substance, and lethal toxin in different preparations would vary to a much greater extent than has been observed in the course of this work.

Attempts to crystallize the toxic protein have so far been unsuccessful. But the criteria of purity described in this and the previous papers could well be applied to a crystalline preparation.

The porphyrins are apparently substances of an acidic nature not combined with the negatively-charged proteins above pH 5.6. Below this pH they are insoluble in water. Since the porphyrins may be readily separated from proteins without any changes in their absorption spectra they are probably not decomposition products of a complex in the toxin preparations. Hosoya and his collaborators (1934) have made similar studies on the porphyrins in anatoxin.<sup>2</sup> The question of whether diphtheria toxin itself shows absorption bands either in the visible or ultraviolet parts of the spectrum has been taken up in recent papers by Levaditi and his co-workers (1934), and by Ottensouser, Krupski, and Almasy (1935). The results were inconclusive. The porphyrins studied in the present work do not appear to be a part of the toxin molecule.

Locke and Main (1928) used iso-electric-point fractionation to separate toxin from bacterial protein but their preparations were considerably denatured, contained much pigment, and no measurements of the completeness of separation were reported.

#### SUMMARY

Methods for separating toxin and bacterial protein by iso-electric-point fractionation are described.

Measurements by the precipitin test of the amount of bacterial protein in purified toxin were based on titrations of the antibac-

<sup>2</sup> In a recent paper Hosoya and his associates (1936) have described a method for the preparation of dried purified diphtheria toxin. In their preparations the M.L.D. is 0.01 mgm. which represents a toxicity only about five times as great as that of dried crude toxin.

terial serum against preparations containing known amounts of bacterial protein. The results indicate that the bacterial protein constitutes 0.5 to 2 per cent of the total protein in the most highly purified preparations.

Slightly denatured toxin is precipitated at a higher pH, and a lower concentration of ammonium sulphate, and is more readily adsorbed on magnesium or aluminum hydroxide than is undenatured toxin.

Evidence is presented that the porphyrins found in crude and purified toxins are not combined with protein but are precipitated near the iso-electric point of toxin and are easily adsorbed on protein precipitates.

The results of this work indicate that the purified protein, which has been freed of all but small traces of impurities detectable by the methods employed, is diphtheria toxin.

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## STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

### IX. TISSUE EXTRACTIVES IN THE GROWTH OF THE DIPHTHERIA BACILLUS<sup>1</sup>

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It was shown in the sixth paper of this series (Mueller, 1935c) that the organic substances present in a tissue extract (liver) which are essential for the growth of a strain of diphtheria bacillus, could be very considerably purified. The method consisted in vacuum concentration of a hot aqueous extract, precipitation with alcohol up to 95 per cent concentration, and removal of the alcohol *in vacuo*. The solution was precipitated with neutral lead acetate, the precipitate discarded and excess lead removed from the filtrate with H<sub>2</sub>S. The resulting material was adsorbed twice with norit charcoal, and the latter boiled out three times with acid 50-per-cent ethyl alcohol. Evaporation *in vacuo* until the alcohol was removed completed the preparation. The resulting concentrate is a clear, light brown limpid solution, acid in reaction and with an unpleasant bitter taste. One cubic centimeter is equivalent to about 85 grams of liver, and contains 41.0 mgm. solids with 3.9 mgm. nitrogen. (The point to which evaporation may be carried is entirely a matter of convenience.) From 0.1 cc. to 0.2 cc. of this solution, added to 10 cc. of otherwise extractive-free media containing suitable amino acids, inorganic salts and a source of energy, i.e., glycerol or sodium lactate, induces maximum growth with practically all the strains of *Coryne-*

<sup>1</sup> The writers are indebted to the Lederle Laboratories for the preparations of liver extract used in these studies.



*bacterium diptheriae* which have been tested. The present paper describes various attempts to isolate the one or more compounds which bring about this effect.

#### CHOICE OF TEST STRAIN

Since the amino-acid requirements for a culture of the Park Williams number 8 strain had been defined (Mueller and Kapnick, 1935) it was thought best to use this organism rather than the strain ('[H Y']') employed in our earlier work with liver extract, as the Park strain is readily available to all laboratories. It turned out that the choice was an unfortunate one, for reversal of the pH often failed to take place when various fractions of the eluate were tested with the result that little growth was obtained, and it was impossible to be sure whether such fractions were completely inactive or whether more abundant growth would have taken place if the reaction had turned alkaline. Meanwhile it had been found that the advantage of general distribution of this strain was illusory for there is a remarkable and surprising difference in growth requirements for cultures called Park 8 obtained from various sources. A forthcoming paper will deal with a study of these differences.

Attempts were made in two directions to overcome the difficulty due to the reaction. In the first place the media were buffered by the addition of 0.25 per cent  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , which resulted in moderate improvement only. Secondly, the replacing of glycerol by sodium acetate or sodium lactate in the usual control formula was attempted with the hope that the base made available in the breaking down of the acid would aid in the reversal of reaction. The latter expedient proved to be extremely satisfactory. In addition, when tested with several strains of the Park 8, and with other more recently isolated cultures, it developed that lactic acid was much more favorable to growth of certain strains than glycerol, even when the question of pH was not involved. The reverse was also true, and the particular Park 8 strain which we had studied in some detail, failed to grow as heavily with sodium lactate as it did with glycerol.

In the course of these experiments on the reversal of reaction,

we encountered a freshly isolated strain of *C. diphtheriae* which grew much more rapidly in the first twenty-four hours than the various Park 8 cultures. With the latter, it was necessary to wait at least 36 to 40 hours before judging grossly of the comparative abundance of growth, and three days to determine the amount for record. With the new strain, on suitable media, abundant growth was present in eighteen hours, and only two days were necessary for complete development. The saving of time made possible by using this culture appears to offset the disadvantage of introducing another non-standard strain. The new culture ("Allen strain"), morphologically exhibits good polar bodies, but shows few club forms. It ferments typically and is virulent for guinea pigs. When freshly isolated, it failed to produce a pellicle on meat infusion-peptone broth even after a considerable number of transplants. Carried during the same period on a medium of the following composition, it formed a well developed pellicle after a few generations.

Casein hydrolysate*.....	1 0	per cent
Cystine..	0 01	per cent
Glutamic acid hydrochloride.	0 5	per cent
Salt mixture†.....	0 25	cc.
Glycerol.....	0 5	per cent
Liver eluate. . .	2 0	per cent

Transfer from this medium to meat infusion-peptone broth did not result in pellicle formation on the latter. The strain has, therefore, been carried in stock culture by daily transplant on the above medium, and has maintained its virulence and fermentative properties unchanged for 16 months. When it was determined to

\* Commercial casein boiled eighteen hours with 10 parts concentrated HCl, evaporated to thick syrup *in vacuo*, taken up in water to make a 20 per cent concentration (calculated on weight of original casein), and stirred cold with sufficient norit charcoal so that the color after filtration is pale yellow.

† Salt mixture:

NaCl.....	20 0	grams
Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O.....	10 0	grams
KH <sub>2</sub> PO <sub>4</sub> .....	1 4	grams
MgCl <sub>2</sub> ·6H <sub>2</sub> O.....	1 2	grams
HCl.....	5	cc.
Water to 100 cc.		

adopt this organism as the test strain, a number of ampules of a young culture were frozen and dried on the Flosdorf-Mudd apparatus. Recently it has been necessary to go back to this material since the passage culture suddenly became quite different in certain of its growth requirements although still virulent and typical in other respects. The dried culture, eleven months old, was living and perfectly characteristic.

#### METHODS

The procedure is described in the earlier papers of this series to which reference is made (Mueller 1935a, b and c, and Mueller and Kapnick 1935). The relative quantities of growth are indicated by the milligrams of nitrogen in the centrifuged and washed bacteria after either two or three days incubation.

#### EXPERIMENTAL

*I. Selective adsorption.* A large number of experiments were carried out in which it was attempted to effect further purification of the eluate by adsorbing with fuller's earth, charcoal or lead sulfide. In general, the results were unsatisfactory, although it was possible to remove considerable inert material, amounting perhaps to a third or more of the total, including practically all the color, by a single adsorption with a small amount of fuller's earth followed by a similar treatment with a small quantity of norit. The activity was removed, however, by further adsorption with either reagent and could not be quantitatively eluted. It appears from these results that some essential component is either destroyed or becomes so firmly attached to the adsorbing material when exposed in a moderately purified form, that it cannot be removed.

On the whole, the results with selective adsorption were so difficult to duplicate that it seemed wise to abandon them and search for a better method.

*II. Various precipitants.* The reagents used were  $\text{HgCl}_2$  in both aqueous and alcoholic solution,  $\text{HgSO}_4$ ,  $\text{Ag}_2\text{SO}_4$  with acid, neutral and alkaline reaction, and  $\text{Ca}(\text{OH})_2$  and alcohol-ether.

The results with the Hg and the Ag salts were not promising.

In general, the active material was not precipitated, although silver in alkaline solution brought down part of it. Moreover, there was usually considerable loss, due perhaps to adsorption on the sulfide precipitates formed in removing the metals.

Of all of the precipitation methods tried, that in which calcium salts were thrown down with alcohol and ether seems to give the most encouraging results. The procedure consisted in adding  $\text{Ca}(\text{OH})_2$  suspension to the eluate until alkaline, then adding 10 volumes each of ethyl alcohol and ether. The precipitate was removed by filtration, and the alcohol and ether removed from the filtrate by vacuum distillation. Although some activity was lost in the precipitate, the greater part of the material passed into the filtrate. A moderate amount of purification could be effected in this way with considerable regularity and the method was utilized in some of the further procedures to be described. On the whole, however, it is doubtful whether enough is to be gained to be of any real value and no detailed protocols of these experiments need be given.

*III. Extraction with immiscible solvents.* The excellent results obtained with butyl alcohol in the separation of amino acids suggested its use for our present purpose. A number of experiments were carried out, of which the following is an example:

*March 6, 1936.* The material used was a calcium-alcohol ether filtrate prepared as described above from a liver eluate made rather differently from the one which has been employed in most of the experiments. In this instance, a liver concentrate (Lederle Solution Liver Extract Parenteral Refined and Concentrated, NNR), and containing considerably more total material than the 95-per-cent alcohol filtrate which we had used, was absorbed on charcoal and eluted with acid alcohol in the same way as the other preparation. The method is described by Subbarow, Jacobson and Fiske (1936).

Ten cubic centimeters of this preparation, of which 1 cc. represented 100 grams liver, were made strongly alkaline by the addition of 1 cc. of 10 N NaOH. The solution was then extracted five times by shaking in a test tube with 10 cc. butyl alcohol, centrifuged in order to hasten the separation, and the butyl alcohol extracts pipetted off. The aqueous residue was then acidified with 2.5 cc. concentrated HCl and the acid solution again extracted five times as before. Each fraction, mixed

with about two volumes of water was neutralized to litmus, the butyl alcohol distilled out *in vacuo*, adding more water as required, and the solutions each brought to a final volume of 40 cc.

Media were prepared as follows and inoculated with the Allen strain. Nitrogen determinations were made on the washed bacteria after about 40 hours growth.

1. Control solution\* 9.6 cc. + NaOH extract 0.4 cc..... 0.74 mgm. N
2. Control solution 9.6 cc. + HCl extract 0.4 cc..... 3.36 mgm. N
3. Control solution 9.6 cc. + Residue extract 0.4 cc.. . . 0.20 mgm. N

Nitrogen determinations on the original material and the three fractions gave the following results:

Ca-alcohol-ether filtrate.....	6.62 mgm./100 grams liver
NaOH extract.....	1 56 mgm./100 grams liver
HCl extract.....	2 23 mgm./100 grams liver
Residue.....	2 24 mgm./100 grams liver
	6 03 Loss about 10 per cent

Experiments of this type invariably gave results of the same sort, and it was evident that nearly two-thirds of the total nitrogenous material of the calcium-alcohol filtrate could be removed with very little loss of activity. The moderate growth with the alkaline butyl fraction and the fact that not infrequently somewhat better growth was obtained by mixing the three fractions together in the media, than by the use of the acid extract alone, may be significant. For the present, the relatively feeble effect of the alkaline extract and the residue have been disregarded.

The next step was to attempt the further purification of the HCl-butyl alcohol extract by shaking out with ether. Surprisingly, this resulted in a separation of the active material into two fractions, neither one alone producing maximal growth, but when mixed, being fully active. The following is a typical experiment:

*March 19, 1936.* A portion of the same calcium alcohol-ether filtrate used in the experiment of March 6 was diluted with three parts of water.

\* Control solution has same composition as formula for stock media, page 155 without the liver eluate, and with 0.1 cc. lactic acid, as sodium lactate, replacing the glycerol. It is so prepared for stock purposes that the quantities required for 10 cc. media are contained in 2.5 cc. of solution. This is diluted as required for use.

One hundred cubic centimeters of this solution were adsorbed by stirring for five minutes with 3.2 grams fuller's earth, filtered, and the filtrate treated in the same way with 1.25 grams norit charcoal. These quantities had previously been shown to remove a considerable amount of inert material and to cause little change in growth stimulating properties. The filtrate was concentrated *in vacuo* to 25 cc.

To 10 cc. of this material was added 1.0 cc. of 10 N NaOH, and the alkaline solution was extracted five times with 10 cc. portions of butyl alcohol. The aqueous portion was then neutralized with concentrated HCl, and 1 cc. additional acid added. Ten extractions with 10 cc. ether were then carried out, followed by five more with 10 cc. butyl alcohol. The acid butyl and the ether extracts were freed from solvent by vacuum distillation, after neutralizing, and made up to 50 cc. each with water. The following experiment was set up in duplicate:

	<i>Mgm. bacterial</i>	
	A	B
The loss, of course, is purely mechanical.		
1. Control solution 9.5 cc. + ether extract 0.5 cc. . . .	1 37	1 60
2. Control solution 9.5 cc. + acid butyl extract 0.5 cc. . .	1 39	1 39
3. Control solution 9.5 cc. + both extracts 0.5 cc. . . .	3 26	2 86

The control solution alone gives a negligible amount of growth. Doubling, or increasing still more the quantity of either fraction, does not give an increase of growth comparable to that produced by the mixture.

Several further experiments indicated that the order of carrying through these procedures was unimportant. The ether extraction could be done on the original eluate or calcium-alcohol-ether filtrate, or the butyl alcohol extraction could be done first, and later, after removing butyl alcohol, the material shaken out with ether. In all cases the separation took place only from an acid solution.

#### DISCUSSION

The comparative ease with which the activity of these preparations is lost by adsorption on various substances leaves any type of purification involving the production of precipitates open to considerable risk. In the course of an investigation of this type there is no means of knowing how many separate substances are

involved in the effect being studied. It is clear that if two or more compounds are required the loss or partial destruction of any one will be followed by greatly diminished growth. In all experiments, therefore, the possibility of a separation into two fractions, each necessary, was kept in mind, but it was only with the employment of extraction with butyl alcohol and ether that facts were observed which indicated that this was really the case.

The observation that a second adsorption could not always be followed by satisfactory recovery of the material, renders such methods of purification on a large scale hazardous. The same applies to precipitation methods. Were it not for this fact, and perhaps even in spite of this drawback, a considerable degree of purification might be accomplished by removal of inert materials by suitable combinations of these methods. Extraction with organic solvents, however, should avoid any such danger and from a number of such small-scale experiments there is every indication that their behavior is uniform and that a satisfactory separation of the activity into two fractions can be made by butyl alcohol and ether with a minimum of loss. Neither of these fractions alone is entirely without activity in promoting a certain amount of growth of the organism, but quantitative experiments in which varying amounts of both fractions are used have indicated clearly that the combined action of the two is essential for optimal growth. The further studies of these two fractions will be the subject of the next paper of this series.

#### CONCLUSIONS

1. The liver eluate previously described can be freed from a small proportion of inactive material by adsorption with either fuller's earth or charcoal.
2. The use of larger quantities of adsorbants results in a partial loss of activity either by destruction or failure of elution.
3. Purification by means of precipitating with heavy metal salts has not proved useful.
4. A certain amount of inert material is removed by precipitation with lime and alcohol and ether.

5. The active materials are extracted from strongly acidified aqueous solution by butyl alcohol but not from an alkaline one.

6. The materials so extracted can be separated into two fractions by repeated extractions with ether; also only from acid solution. Neither of these fractions alone shows the full effect of the combination of the two in stimulating growth of the diphtheria bacillus. These fractions are being further studied.

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## STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

### X. PIMELIC ACID AS A GROWTH STIMULANT FOR *C. DIPHTHERIAE*

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In the preceding paper (Mueller and Subbarow, 1937) it was shown that the constituents of a tissue extract (liver) essential for heavy growth of a strain of *Corynebacterium diphtheriae* were at least two in number, and that they could be separated from each other by ether extraction of an acidified concentrate. Since the amount of total material passing into the ether was only a small fraction of that remaining in the aqueous phase, and further, because the solubilities of the former gave a definite clew as to its probable chemical nature (organic acid or phenol), it seemed wise to attempt the isolation of this substance first, leaving a further study of the other, perhaps still multiple, factor until later.

It is clear that in studies of this sort, involving an unknown number of variables, it is difficult, and yet essential so to plan the work that a control solution shall be available, and susceptible to ready duplication, which will give an unmistakable indication of the presence or absence of a fraction under investigation over a considerable period of time. After various preliminary experiments which indicated clearly that the phenomenon to which reference is made could be produced regularly, and that the ether-soluble material was probably an organic acid and not a phenol, a control solution which seemed likely to be adequate to the needs of the work was arranged. The basis for this was the formula previously used, a casein hydrolysate,—enriched by cystine and glutamic acid both of which are present in less than optimal con-

centration when one per cent of casein is employed,—together with suitable inorganic salts and sodium lactate. To this had to be added the ether insoluble fraction of the liver concentrate. A whole protein hydrolysate is used, rather than pure amino acids, in spite of the certain presence of unknown substances, for two reasons. First, should it be essential for any reason to employ a different test strain in the course of the work, a considerable delay might be anticipated in arriving at a suitable amino-acid formula, because of the considerable differences between various cultures of the diphtheria bacillus. In the second place, the expense involved is negligible as compared with that connected with the extended use of pure amino acids.

A uniform supply of casein hydrolysate, adequate for a long series of experiments, was assured by hydrolyzing several pounds of casein with concentrated HCl, evaporating to a thick gum, then dissolving in water to a concentration corresponding to 20 per cent of the protein and treating with norit charcoal at room temperature until only a pale straw color remained after filtration. Toluol was added and the material kept in the cold room, although such a preparation will keep for long periods at room temperature without preservative because of the highly acid reaction.

The ether-insoluble liver fraction was prepared from the calcium-alcohol filtrate described in the last paper (IX), using a quantity corresponding to 60 kgm. of liver tissue. The details of the preparation need not be given. Essentially it consisted in butyl-alcohol extraction of the acidified solution, the transfer of the extracted material to water followed by removal of the ether-soluble fraction. The remaining material, thoroughly dried, was extracted with absolute methyl alcohol, and the solution precipitated fractionally with ether. The most active fraction (the third) was dissolved in 50 cc. hot absolute ethyl alcohol and after cooling over night in the ice box was filtered from a small amount of separated solid. Of this solution 0.01 cc. in 10 cc. of medium equivalent to 12 grams of fresh liver tissue, proved to be adequate for heavy growth in the presence of the other constituents of the

control solution plus a sufficient amount of the ether-soluble fraction. The solution contained 0.0428 gram material per cubic centimeter. The quantity was sufficient for 5000 test lots of media. At the conclusion of the experiments about half of the solution remains. Reference is made to this solution in the protocols as "4b."

The course of the experiments may be traced briefly, after which representative experiments will be presented to illustrate the more important steps.

Assuming the active material to be one or more organic acids, esterification, followed by vacuum distillation was first carried out, and found to yield a distillate active after hydrolysis. Through the courtesy of Pappenheimer (1935), we were able to test some of his "sporogenes vitamin" having similar properties but obtained no particular effect from its use. Having in mind, however, his finding of the substance in urine, as well as in tissue and yeast extracts, we examined the urine of several different species of animals for the diphtheria ether-soluble fraction. The experiments indicated the presence of material which adequately replaced the liver acid. Particularly in the urine of the horse and cow, it evidently occurred in greater concentration than in fresh liver tissue. This active material, too, proved to be ether-soluble (from acid solution), and to be esterifiable and to distill in vacuum presumably as the ester. Through the courtesy of several commercial houses which manufacture theelin from urine, and of various experimental workers in the field, certain concentrates containing the organic acids of urine were tested, but none were found suitable for use. Suspecting wide differences in partition of the substance between water and various organic solvents, the point was tested, and butyl alcohol, ethyl acetate and ethyl ether proved able to extract the material easily, while benzene, chloroform and petroleum ether failed to remove appreciable amounts.

Preliminary experiments on concentrates prepared from small quantities of human and horse urine indicated that the distilled esters were a mixture of several substances, yielding on hydrolysis

a discouraging oily material, and that the greatest hope of success lay in working through sufficient material to carry out adequate fractional distillations.

One hundred gallons of cows' urine, calculated to yield between 50 and 100 grams of distilled methyl ester were therefore processed. A detailed account of the method from the chemical point of view will be presented elsewhere. It included preliminary extraction of the acidified urine with butyl alcohol, instead of ether, removal from the solvent with  $\text{NaHCO}_3$  solution, separation of a large amount of hippuric acid by acidification and crystallization, and then passing back and forth between butyl alcohol and aqueous sodium bicarbonate, gradually reducing the volume and at each transfer eliminating considerable quantities of inert material. A barium-hydroxide alcohol precipitation greatly aided the purification, the active substance appearing in the filtrate from the bulky precipitate. Esterification was finally effected in absolute methyl alcohol with  $\text{HCl}$ , a further purification at this stage resulting from the removal of non-ether-soluble esters (phenaceturic acid, etc.) and non-esterifiable material by extraction with  $\text{NaHCO}_3$  and finally weak  $\text{NaOH}$ .

The resulting esters, after drying, yielded 83 grams vacuum distillate up to  $163^\circ\text{C}$ ., using an oil pump vacuum of less than 1 mm. It was separated empirically into five fractions by means of a Fischer triangle as it passed over. Eighty grams of dark brown residue remained in the flask at the end of the distillation. All the fractions showed activity, but this was most pronounced in the lower-boiling portions. Various types of redistillation were tried on small lots, particularly on fractions resulting from earlier small scale experiments. It was found that the material was surprisingly stable, and would withstand even atmospheric pressure distillation, passing over with no evidence of decomposition and fully active at about  $250^\circ$ , but still too impure to deal with the regenerated acids.

At this point we learned of a small specially constructed vacuum distilling column designed and being used by Dr. David Rittenberg of the Department of Biochemistry at the College of

Physicians and Surgeons in New York, with which he was able to separate almost quantitatively small amounts of substances with boiling points only a few degrees apart. We were fortunate in being able to enlist Dr. Rittenberg's cooperation and not only learned from him how to construct and operate his column, but actually watched him run a distillation on one of our active fractions. From about three grams of our esters he separated ten fractions, and subsequent test showed a very satisfactory degree of concentration in the middle boiling portions.

After setting up an apparatus similar to his, the most active of the total ester fractions were systematically distilled, and the most active of the resulting fractions re-distilled. Tests indicated the highest activity in two adjoining fractions in about the middle of the range covered. The lower boiling of these remained a clear, thick syrup even when cooled in carbonic ice and alcohol, whereas the higher one set to a solid mass of fine crystals. It was decided to hydrolyze this latter fraction, amounting to 2.5 cc. About half of it was boiled with alcoholic NaOH, acidified, evaporated to dryness and extracted with ether. Removal of the ether left a definitely granular material mixed with some oil. In this impure form difficulty was next experienced in finding a suitable solvent with which to attempt recrystallization. The substance proved to be practically insoluble in some, and much too readily soluble in other solvents. Dibutyl ether was finally employed and from this, crystallization readily took place, the material being practically insoluble at room temperature, but dissolving easily on heating. After three recrystallizations the melting point appeared to be constant at  $104^{\circ}$  to  $105^{\circ}$ , and granular white crystals, 1 to 2 mm. in diameter were formed. The yield was 0.3 gram.

The crystals proved to be highly active in growth tests. Titration with  $N/100$  NaOH, semi-micro combustions, micro-molecular weight determinations and the preparation of the phenylphenacyl ester of the acid and a molecular weight determination on this gave results consistent with the formula  $C_8H_{10}(COOH)_2$ . The dibasic acid of this formula, pimelic acid, is stated to melt at

105°, as did our crystals. Mixed melting points with Eastman's pimelic acid showed no lowering, and growth tests with the latter were in every way identical with those carried out on our isolated crystals. The effect becomes apparent at a concentration of about 0.005γ of pimelic acid and reaches a maximum at about 0.025γ, per cubic centimeter of culture medium. An excess of the substance produces no further change even up to a concentration of 1 per cent.

The next lower boiling fraction of esters, showing almost as great an activity as the one used above was worked up in the same way, and from this, also, pimelic acid was readily obtained. More impurity was present, and it was necessary to recrystallize repeatedly from butyl ether, and finally twice from water—with considerable loss—before good crystals with the correct melting point were obtained. The other material in this fraction was not further investigated.

From an earlier preparation of esters on a smaller scale there had been obtained a small amount of a crystalline hydrazide, from which it was possible to prepare and recrystallize a substance which proved to be azelaic acid,  $C_7H_{14}(COOH)_2$ . A growth test with this material proved negative, and since the sticky, non-crystalline impurities were highly active, azelaic acid was not investigated further at that time. After the isolation of pimelic acid, however, all the dibasic acids up to and including azelaic were carefully tested. None of these were found to have an effect in any way comparable with that of pimelic acid.

It should be added that a certain amount of growth acceleration was produced by another of the ester fractions, one of the lower boiling ones. This material is definitely different from the esters of the dibasic acid. It has a highly aromatic odor, somewhat suggestive of that of methyl benzoate, and has given crystals which are probably phenyl acetic acid. The effect of these crystals, as of phenyl acetic acid itself, is irregular, heavy growth not being produced in any case, and adding them to pimelic acid gives no better growth than the pimelic acid alone.

There is no indication at present as to the mechanism for this peculiar action of a compound which, as far as the writer can

learn, has not previously been encountered in biological material. Its action is not limited to our test strain (Allen), but is manifested with at least two strains of Park 8, one from the National Institute of Health, the other from the Alabama State Department of Health. Whether it will prove to stimulate the growth of bacteria other than the diphtheria bacillus has not been investigated. Suggestive, however, are the findings of Snell, Tatum and Peterson (1937) and Wood, Tatum and Peterson (1937) that unidentified organic acids are necessary for growth of certain lactic and propionic bacteria. Professor Peterson has kindly tested for the writer a still impure, but active preparation containing pimelic acid, on his lactic strain and found it to be without effect. Dr. Tatum, now in Utrecht, writes that the acids concerned in growth of his propionic organisms are extracted with ether only with the greatest difficulty. Probably further work will indicate whether these higher dibasic acids have a more general biological significance.

It remains to express again the writer's sincere appreciation of the cooperation given so freely by Dr. Rittenberg. Without his assistance the completion of this problem would have been infinitely more difficult.

#### EXPERIMENTAL

The control solution for 10 cc. of medium consisted of the following materials, and unless otherwise noted, remains constant in subsequent experiments:

Casein HCl hydrolysate.....	0.100	gram
Cystine.....	0.001	gram
Glutamic acid hydrochloride.....	0.050	gram
NaCl.....	0.050	gram
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ .....	0.025	gram
$\text{KH}_2\text{PO}_4$ .....	0.0035	gram
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ .....	0.003	gram
Lactic acid (as Na salt).....	0.10	cc.

The "Allen" strain (paper IX) was used as test organism, and the amount of growth is given in terms of milligrams of bacterial nitrogen after 48 hours' growth.



*I. The ether-soluble factor from liver extract may be esterified and distilled*

Six hundred cubic centimeters of the same calcium-alcohol-ether filtrate of a liver concentrate used in the experiment of March 6, 1936, and described in the previous paper, were employed. This was equivalent to approximately 60 kgm. of fresh liver tissue. It was diluted to 2400 cc., stirred for five minutes with 75 grams Fuller's earth and filtered, and then treated similarly with 30 grams norit charcoal, and finally with 10 grams more norit. After the third filtration the solution was only slightly yellowish in color. It was concentrated in vacuo to 175 cc.

After making alkaline by the addition of 17.5 cc. of 10 N NaOH it was extracted five times with 200 cc. butyl alcohol, then neutralized with concentrated HCl, and 17.5 cc. excess added. Ten extractions with 200 cc. of peroxide-free ether were then carried out, followed by five with 200 cc. butyl alcohol.

The combined wet ether extracts were evaporated to about 30 cc. in vacuo. The residue, largely water, was transferred to a 500-cc. boiling flask; frozen around the wall in dry ice and transferred to the Flosdorf-Mudd drying apparatus, and the water thoroughly removed by oil pump vacuum for 18 hours. The residue consisted of 1.3 grams of a mixture of crystals and a thick yellow oil. The latter was largely removed by successive washings with small amounts of ether, and was shown to contain the active substance. The crystals (succinic acid?) were without activity, and weighed about 0.15 gram.

The ether was removed from the solution of the oily material, which was then dissolved in 10 cc. absolute methyl alcohol. To this was added 1.0 cc. of a 30 per cent solution of dry HCl in methyl alcohol. The mixture was heated briefly to boiling, and allowed to cool slowly and stand without further heating for two hours. As much alcohol and HCl as possible were then removed by vacuum distillation, and the residue dissolved in ether and washed successively with saturated  $\text{NaHCO}_3$ , 0.5 per cent NaOH, and water. The ether solution of the esters was then dried with  $\text{Na}_2\text{SO}_4$ , transferred in small portions to a Wassermann tube and the ether removed in a current of warm dry air. The residue, presumably methyl esters, weighed 0.225 gram.

After some preliminary experiments using a few milligrams of the material, the remainder, of perhaps 0.2 gram of esters in the small test tube was subjected to vacuum distillation. The test tube was sealed to a long narrow glass tube which was then bent to form a narrow U-shaped trap. The esters were heated in an oil bath to 120° for about an hour, the trap being cooled with dry ice, and a vacuum of less than 1 mm. applied from an oil pump. Distillate collected both in the trap and in the air-cooled section of the apparatus. No attempt was made to secure different fractions. After releasing the vacuum and bringing the apparatus to room temperature, the portion of trap and connecting arm containing the distillate was cut away from the rest of the apparatus. About one-third of the material appeared to have distilled. It was a water-clear liquid with a very faint odor somewhat resembling that of malonic ester.

About 0.0002 gram of the ester was transferred to 10 cc. N/1 NaOH and heated fifteen minutes in the water bath to effect saponification, and growth tests prepared as follows:

	mgm. N
1. Control + HCl-butyl alcohol extract . . . . .	1 06
2. Control + HCl-butyl alcohol extract + saponified esters 0.1 cc. . . . .	1 62
3. Control + HCl-butyl alcohol extract + saponified esters 1.0 cc. . . . .	2 86

## *II. The ether-soluble factor is not identical with "Sporogenes vitamin"*

Through the kindness of Dr. Pappenheimer a small quantity of the distilled ester of this material was made available for testing. Two milligrams were saponified by autoclaving for ten minutes at 10 pounds pressure with 2 cc. N/1 NaOH, the following test carried out:

	mgm. N
1. Control + 4b <sup>1</sup> + 0.1 mgm. sporogenes vitamin ester.....	1.90
2. Control + 4b + 0.01 mgm. sporogenes vitamin ester.....	0 66
3. Control + 4b + 0.001 mgm. sporogenes vitamin ester.....	0.66
4. Control + 4b + 0.0001 mgm. sporogenes vitamin ester..	0 74
5. Control + 4b + 0.00001 mgm. sporogenes vitamin ester.....	0.68
6. Control + 4b.....	0 64

<sup>1</sup> See page 165.

Since the sporogenes vitamin manifests activity in quantities of small fractions of a gamma, and since from *I* it is evident that the liver acid is active in quantities of less than 0.01 mgm. even in an impure form, it is clear that the two are not identical. The growth with 0.1 mgm. may have been caused by a trace of impurity in the sporogenes preparation or, the substance itself may have an effect of its own at this concentration.

*III. The ether-soluble fraction of liver extract can be replaced by urine*

	mgm. N
1. Control + 4b + .....	0 70
2. Control + 4b + human urine 1.0 cc.....	3 44
3. Control + 4b + human urine 0.1 cc.....	3 56
4. Control + 4b + human urine 0.01 cc.....	1 30
5. Control + 4b + mare's urine 0.1 cc.....	3.16
6. Control + 4b + mare's urine 0.01 cc.....	2.16
7. Control + 4b + mare's urine 0.001 cc. ....	0 70
8. Control + 4b + cow's urine 0.1 cc.....	3 24
9. Control + 4b + cow's urine 0.01 cc. ....	3 26
10. Control + 4b + cow's urine 0.001 cc. . .	1 04

*IV. Extraction of active substance from urine by various solvents*

Cow's urine was acidified by adding 25 cc. concentrated  $H_2SO_4$  per liter, and after standing over night, a small amount of kieselguhr was added, and the material filtered by gravity through a pleated filter paper. Twenty-five-cubic centimeters portions of the filtrate were thoroughly shaken with 25 cc. quantities of the following solvents, with results as shown:

1. Butyl alcohol	Considerable material extracted
2. Ether	Considerable material extracted
3. Ethyl acetate	Considerable material extracted
4. Chloroform	Small amount of material extracted
5. Benzene	Small amount of material extracted
6. Petroleum ether	Practically nothing extracted

After evaporation of the solvent, the extracted materials were taken up in 25 cc. water each, with a little NaOH to effect solution.

Growth tests showed the first three extracts to be highly active, the others completely inactive.

*V. The preparation and distillation of esters from 100 gallons of cow's urine*

This was carried out in daily lots of 10 gallons, acidifying with  $\text{H}_2\text{SO}_4$ , filtering, and extracting three times with about one-third volume of butyl alcohol, using 5-gallon bottles for the procedure. The butyl alcohol was in turn extracted with a suspension of  $\text{NaHCO}_3$  in water, to alkaline reaction, with thorough and prolonged mixing by means of an air current, and the aqueous solution of sodium salts of the acids combined from day to day, until the entire amount had gone through this stage. In this way there was obtained about 10 gallons of concentrate. The further purification of this material will be described in detail in another place. Eventually, there was obtained 120 grams of dry methyl esters ready for distillation, and possessing a satisfactory degree of activity in growth tests.

Distillation was carried out from a 500-cc. Claissen flask, using a Fischer triangle, dry ice trap, and oil pump vacuum of less than 1 mm. Hg. The following fractions were taken:

	TEMPERATURE OF VAPORS	TEMPERATURE OF OIL BATH	WEIGHT OF FRACTION
	<i>degrees</i>	<i>degrees</i>	<i>grams</i>
I	81-84	85-114	15
II	93-109	135	19
III	110-130	155	25
IV	130-149	175	11
V	158-163	195	13

A few milligrams of each of these distillates, as well as of the residue, were weighed out and saponified 1 mgm per cubic centimeter with  $N/1$   $\text{NaOH}$ . An abbreviated portion of the test with these solutions shows the following comparative effects on growth:

	<i>mgm. N</i>
1. Control + 4b + distillate I, 1.0γ.....	1 46
2. Control + 4b + distillate II, 1.0γ.. . . .	1.58
3. Control + 4b + distillate III, 1.0γ.. . . .	1.14
4. Control + 4b + distillate IV, 1.0γ.....	0 98
5. Control + 4b + distillate V, 1.0γ.....	0 88
6. Control + 4b + residue, 1.0γ (nitrogen not done, no increase).	
7. Control + 4b.....	0 70

Fractions I and II were re-distilled separately under atmospheric pressure, mixing the distillates of the second with those of the first having corresponding boiling points. Distillation was carried out slowly, from an oil bath. The following fractions were obtained:

	VAPOR TEMPERATURE	WEIGHT OF DISTILLATE	
		From fracture I	From fracture II
	<i>degrees</i>	<i>grams</i>	<i>grams</i>
A	198-210	2.5	
B	210-220	5.9	
C	220-225	2.2	
D	228	0.8	
E	233-245	1.4	3.0
F	245-255	0.5	5.0
G	255-265		6.3
H	265+ (both to 300°)		4.5

Residue 3 to 4 grams.

Growth tests made on these fractions, after hydrolysis, as before, showed little activity in fractions A and B, and in the residue. Values for the others resulted thus;

	<i>mgm. N</i>
1. Control and 4b + fraction C, 1.0γ. ....	1.34
2. Control and 4b + fraction D, 1.0γ. ....	1.86
3. Control and 4b + fraction E, 1.0γ. ....	2.30
4. Control and 4b + fraction F, 1.0γ. ....	2.24
5. Control and 4b + fraction G, 1.0γ. ....	1.96
6. Control and 4b + fraction H, 1.0γ. ....	1.76
7. Control and 4b....	0.62

The following distillation was carried out by Dr. Rittenberg on 3.1 grams of fraction F. The description of the apparatus will be published shortly by Dr. Rittenberg in connection with his own studies. It consists essentially in a very slow vacuum distillation with the use of an effective fractionating column. Under a vacuum of about 0.04 mm. Hg the following fractions were obtained in about seven hours of actual distillation:

	TEMPERATURE OF VAPORS	APPROXIMATE QUANTITIES
	<i>degrees</i>	<i>cc.</i>
1	? (low)	0.7
2	? (low)	0.3
3	50-74	0.4
4	72	0.2
5	78-80	0.3
6	82	0.3
7	80	0.15
8	86	0.2
9	87	0.1
10	Residue	0.4

These distillates were entirely colorless liquids, the first four of which became thick when cooled in dry ice and alcohol but showed no evidence of crystallizing, whereas the later fractions and the residue solidify to a mass of white crystalline material, quickly melting again at room temperature. The first two fractions had a definitely aromatic odor, whereas the others were almost odorless.

Growth tests gave the following results:

	<i>mgm. N</i>
1. Control + 4b + fraction 1, 1.0γ.....	1.88
2. Control + 4b + fraction 2, 1.0γ.....	2.60
3. Control + 4b + fraction 3, 1.0γ.....	2.58
4. Control + 4b + fraction 4, 1.0γ.....	3.12
5. Control + 4b + fraction 5, 1.0γ.....	3.26
6. Control + 4b + fraction 6, 1.0γ.....	lost
7. Control + 4b + fraction 7, 1.0γ.....	2.70
8. Control + 4b + fraction 8, 1.0γ.....	2.48
9. Control + 4b + fraction 9, 1.0γ.....	2.18
10. Control + 4b + residue, 1.0γ.....	2.22

Growth in tube 6 appeared as good as in tubes 4 and 5, and there has consequently been a very considerable concentration of active material into fractions 4, 5 and 6.

The further separation of the bulk of the esters obtained by the atmospheric pressure distillation by means of an apparatus similar to Dr. Rittenberg's need not be given in detail. Beginning with the lowest boiling, each fraction in turn was distilled, using a vacuum of 7 mm. because of the comparatively low boiling

point, with an oil pump vacuum, and separated into several smaller ones. Those of similar boiling points and high activity resulting from different atmospheric pressure fractions were combined and again carefully fractionated. At last a fraction of about 2.5 cc. was obtained boiling between about  $110^{\circ}$  and  $117^{\circ}$  at 7 mm. pressure, which was the lowest boiling fraction to solidify when immersed in dry ice, and which corresponded in a general way with fraction 5 of Dr. Rittenberg's distillation.

The test on this fraction, after hydrolysis, follows:

	<i>mgm. N</i>
1. Control + 4b and active fraction, 1.0 $\gamma$ .....	2.88
2. Control + 4b and active fraction, 0.5 $\gamma$ .....	3.11
3. Control + 4b and active fraction, 0.25 $\gamma$ .....	2.82
4. Control + 4b and active fraction, 0.1 $\gamma$ .....	2.30
5. Control + 4b and active fraction, 0.05 $\gamma$ .....	2.27
6. Control + 4b and active fraction, 0.025 $\gamma$ .....	1.72
7. Control + 4b and active fraction, 0.01 $\gamma$ .....	1.08

#### VI. The active substance is pimelic acid

Half of this fraction yielded 0.3 gram pimelic acid. (Details of analyses, etc., to be published elsewhere.)

The following experiment, put up in duplicate, compares the effect of the natural pimelic acid, isolated from cow's urine with the Eastman synthetic product.

	<i>A</i>	<i>B</i>
1. Control + 4b + natural pimelic acid, 1.0 $\gamma$ .....	4.08	4.05
2. Control + 4b + natural pimelic acid, 0.5 $\gamma$ .....	3.96	4.05
3. Control + 4b + natural pimelic acid, 0.25 $\gamma$ .....	3.61	3.51
4. Control + 4b + natural pimelic acid, 0.1 $\gamma$ .....	2.48	2.28
5. Control + 4b + natural pimelic acid, 0.05 $\gamma$ .....	1.78	1.81
6. Control + 4b + natural pimelic acid, 0.025 $\gamma$ .....	1.68	1.63
7. Control + 4b + natural pimelic acid, 0.01 $\gamma$ .....	1.46	1.44
8. Control + 4b + synthetic pimelic acid, 1.0 $\gamma$ .....	4.01	3.93
9. Control + 4b + synthetic pimelic acid, 0.5 $\gamma$ .....	3.85	3.76
10. Control + 4b + synthetic pimelic acid, 0.25 $\gamma$ .....	3.58	3.54
11. Control + 4b + synthetic pimelic acid, 0.1 $\gamma$ .....	2.14	2.24
12. Control + 4b + synthetic pimelic acid, 0.05 $\gamma$ .....	1.94	1.80
13. Control + 4b + synthetic pimelic acid, 0.025 $\gamma$ .....	1.52	1.62
14. Control + 4b + synthetic pimelic acid, 0.01 $\gamma$ .....	1.49	1.24
15. Control + 4b.....	1.68	1.68

*VII. The action of pimelic acid is not common to related dibasic acids*

To determine this point, a number of experiments have been carried out similar to the one which follows:

1. Control + 4b + oxalic acid, 1.0γ.....	1 08
2. Control + 4b + malonic acid, 1.0γ.....	1 18
3. Control + 4b + succinic acid, 1.0γ.....	1 06
4. Control + 4b + glutaric acid, 1.0γ.....	1 00
5. Control + 4b + adipic acid, 1.0γ.....	1 92 <sup>2</sup>
6. Control + 4b + pimelic acid, 1.0γ.....	3 91
7. Control + 4b + suberic acid, 1.0γ.....	1 11
8. Control + 4b + azelaic acid, 1.0γ.....	1 14
9. Control + 4b .....	1 10

*VIII. The effect of pimelic acid is not limited to the Allen strain, but extends to the Park 8 strain*

The following experiment was set up, using exactly the same control as for the Allen strain, except that 1 mgm. tryptophane was added to each tube. Two strains of Park 8 which differ considerably from each other in certain cultural requirements were used, one from the National Institute of Health in Washington, the other from the Alabama State Department of Health. These cultures had been recently received from the two laboratories, and kept by daily transfer on peptone infusion broth at 34° until used.

	ALABAMA STRAIN	NATIONAL INSTITUTE STRAIN
Control + pimelic acid 10.0γ.....	2 74	3.08
Control.....	1.94	1.19

### CONCLUSIONS

1. The acid-ether extractable substance from liver which stimulates the growth of the diphtheria bacillus can be replaced by a substance present in urine showing similar properties.

<sup>2</sup> Further tests with adipic acid failed to confirm this result.



2. From the latter source it has been isolated in pure form and identified as pimelic acid,  $C_8H_{10}(COOH)_2$ .

3. The effect on growth of synthetic pimelic acid is identical with that of the natural substance, becoming evident at a concentration of about 0.005 gamma per cubic centimeter of medium, and reaching a maximum at approximately five times this amount. Further increase, up to 1 per cent, has no further effect and carries no inhibition.

4. Azelaic acid,  $C_9H_{14}(COOH)_2$  has also been isolated from the urine preparation, but shows no effect on growth of the diphtheria bacillus, nor do any of the other simple dibasic acids from oxalic up to suberic,  $C_8H_{12}(COOH)_2$ .

5. So far as can be learned, pimelic acid has not previously been recognized as occurring in biological, material, and azelaic acid is not listed as a normal constituent of urine.

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# THE IMPORTANCE OF ENRICHMENTS IN THE CULTIVATION OF BACTERIAL SPORES PREVIOUSLY EXPOSED TO LETHAL AGENCIES

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Although the elemental food requirements of all microorganisms may be considered identical, the form in which the essential elements can be utilized by the various species is subject to almost infinite variation. Literally hundreds of different culture media have been proposed, many of which find application because they favor or prevent the growth of particular species or related groups. That the individual organisms which make up a pure culture may also vary widely in their metabolic requirements is apparently not recognized by many bacteriologists. The published reports of many investigators dealing with bacterial resistance and the cultural methods employed by a large number of our control laboratories show how little this fundamental fact affecting the nutrition of bacteria is understood.

It is the purpose of the present paper to show that bacterial spores which survive destructive physical or chemical influences are much more exacting in their nutritional requirements than spores not subjected to such treatment. Evidence is presented which indicates that this phenomenon is not restricted to spores but applies to vegetative forms as well.

## EXPERIMENTAL

### *Cultures and methods*

Of the cultures used *Bacillus subtilis*, *Bacillus cohaerens*, and *Bacillus albolactis* were obtained from The American Type Culture Collection, CC is an unidentified species isolated from spoiled evaporated milk. The spores were produced on infusion agar

slopes. After one to six weeks' incubation, the growth was washed off with distilled water, filtered through cotton, and centrifuged. The supernatant fluid was then completely poured off and the spores resuspended in distilled water, following which they were again centrifuged and resuspended as before. To insure thorough removal of the medium and culture products, the washing process was performed three times. The suspensions were then examined microscopically, and if spore clumps were present these were broken up by vigorous shaking with sterile washed sea sand. The concentrated stock suspensions thus prepared were plated for purity and enumeration and held at room temperature until used. The test spore suspensions in distilled water contained from one to two million viable spores per ml.

The suspensions were heated in pyrex tubes in a stirred glycerin bath equipped with a thermo-regulator which maintained a temperature of  $98^{\circ}\text{C.} \pm 0.5^{\circ}\text{C.}$  The heated suspensions were cooled to  $20^{\circ}\text{C.}$  immediately after their removal from the bath.

The spores were exposed to  $\text{HgCl}_2$  in aqueous solution containing 0.05 per cent of the salt. After their exposure the spores were recovered by centrifugation and washed twice with sterile distilled water.

Ultra-violet light was supplied by a cold quartz mercury-vapor lamp of the official applicator type. The power consumption of this burner is about 9 watts and approximately 95 per cent of the rays emitted are at the line 2537A. Four milliliters quantities of the spore suspension at  $30^{\circ}\text{C.}$  were irradiated in a small quartz flask placed four inches from the burner. The flask was equipped with a quartz stirrer which provided rapid and uniform agitation. After exposure to light, heat or  $\text{HgCl}_2$ , equal portions of the suspension were poured with 15 ml. of agar of the desired composition. The plates were counted after 48 hours' incubation at the optimum temperature of the organism. A magnifying glass was used in counting and the figures represent the averages of triplicate plates. After the initial count was recorded, the plates were returned to the incubator and examined one week later to preclude the possibility that observed differences were due to rate of development rather than capacity to develop.

Six media of the following compositions were used:

1. Nutrient agar: 0.5 per cent peptone (Difco), 0.3 per cent beef extract (Liebig's), 0.5 per cent sodium chloride, 1.5 per cent agar (granulated).

2. Nutrient agar reinforced with one drop of sterile defibrinated cows' blood per plate.

3. Nutrient agar reinforced with 0.3 cc. of sterile 10 per cent glucose solution per plate.

4. Nutrient agar reinforced with 0.3 cc. of sterile 10 per cent yeast extract (Difco) solution per plate.

5. Infusion agar in which the beef extract of the above nutrient agar was replaced by beef infusion made by extracting 500 grams of lean beef with 500 ml. water, heating, filtering and using at the rate of 500 ml. per liter.

6. Tomato-juice milk-powder agar containing in addition to the standard quantities of peptone and beef extract, 5 per cent of tomato juice, 0.5 per cent milk powder, and 0.15 per cent Sörensens' air-dried phosphate. ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ).

#### INFLUENCE OF ULTRA-VIOLET LIGHT UPON THE GERMINATION OF SPORES

In table 1 is shown the number of survivors germinating on different nutrient media when the spores of four species were exposed to ultra-violet light sufficient to kill all but a relatively small proportion. In the first column under each species is also shown the number of colonies developing from the untreated spores plated on the same series of media, at the same time. From this table it may be seen that nutrient agar was much less favorable to the germination of the irradiated spores than to the germination of those not so treated. With two species, namely, *B. subtilis* and CC, enrichments had considerable influence upon the viability of the untreated spores but this effect was always much less than that observed after ultra-violet irradiation. Blood seems to be particularly effective as enrichment for irradiated spores. The effect of yeast extract not only in this but in nearly all experiments was to somewhat reduce rather than to increase the number of germinating spores. The toxicity of yeast

extract was further shown by the fact that combinations of this extract with glucose, blood and other enrichments materially reduced the viable count. It was not possible to increase the count significantly by increasing the quantity of glucose or blood, or by combination of the more effective enrichments. Increasing the concentration of the peptone and beef extract in the nutrient agar formula, likewise failed to increase the total count obtained with this medium.

TABLE 1

*The ability of bacterial spores to germinate in different nutrient media before and after lethal exposures to ultra violet light*

MEDIA	NUMBER OF VIABLE SPORES PER MILLILITER							
	<i>B. cohaerens</i>		<i>B. subtilis</i>		CC		<i>B. albolactis</i>	
	Untreated	Irradiated	Untreated	Irradiated	Untreated	Irradiated	Untreated	Irradiated
	0000 omit- ted		0000 omit- ted		0000 omit- ted		0000 omit- ted	
Nutrient agar.....	134	22	88	55	45	62	47	86
Nutrient agar plus blood .....	140	225	123	315	122	379	51	239
Nutrient agar plus glucose ..	120	64	218	154	137	153	47	83
Nutrient agar plus yeast.....	131	16	47	51	25	54	37	84
Infusion agar.....	142	75	237	171	148	187	41	156
Tomato juice milk powder agar....	149	25	232	88	145	124	34	49

#### INFLUENCE OF HEAT UPON THE GERMINATION OF SPORES

When the spores of the same species were exposed to destructive heat and subsequently cultivated on different media, the results shown in table 2 were obtained. As in the preceding experiment, spores which outlived the treatment were much usually more fastidious in their growth requirements than those derived from the same culture protected from destructive action. The differences observed here in favor of enrichments were much greater than those noted with irradiated spores. Glucose was usually more effective than blood in the cultivation of heat-surviving spores, although the reverse was true in the subculture of irradiated spores. Glucose enrichment not infrequently increased

the number of viable heated *B. cohaerens* spores one hundred fold. Infusion or tomato milk powder may under some conditions equal or excel glucose as enrichments for the cultivation of heated spores.

The need for the application of the enrichment principle to resistance studies is obvious. Under the conditions described above, the thermal death time of *B. cohaerens* spores was 25 minutes when nutrient agar was used as the subculture medium;

TABLE 2

*The viability of bacterial spores in different nutrient media before and after lethal exposures to heat*

MEDIA	NUMBER OF VIABLE SPORES PER MILLILITER							
	<i>B. cohaerens</i>		<i>B. subtilis</i>		CC		<i>B. albolactis</i>	
	Untreated	Heated	Untreated	Heated	Untreated	Heated	Untreated	Heated
	0000 omitted		0000 omitted		0000 omitted		0000 omitted	
Nutrient agar.....	134	40	88	80	45	15	47	157
Nutrient agar plus blood	140	345	123	350	122	160	51	320
Nutrient agar plus glucose ..	120	2100	218	521	137	511	47	210
Nutrient agar plus yeast ....	131	34	47	70	25	6	37	135
Infusion agar. ....	142	1350	237	480	148	440	41	390
Tomato juice milk powder agar...	149	1320	232	400	124	420	34	280

with glucose agar the thermal death time was 39 minutes, an extension of over 50 per cent.

Table 3 shows the results obtained when *Escherichia coli* was cultivated with and without enrichment, before and after exposure to irradiation and heat. The organisms were recovered from an 18-hour broth culture prepared and exposed as were the spore suspensions previously described. Enrichment in one instance increased the number of colonies which developed on nutrient agar almost nine fold, although such growth-promoting substances were practically without effect on the unexposed organisms. Other enrichments were somewhat less effective, although all except yeast extract yielded a much higher count than plain nutrient agar.

Table 4 shows that many spores which survive rigorous chemical treatment, in common with irradiated and heated spores, require enrichments for their germination.

The relative proportion of *B. cohaerens* spores previously irradiated or heated which grow on different media depends largely on the relative proportion of spores which survive the destructive

TABLE 3

*Viability of E. coli in different media before and after lethal exposures to irradiation and heat*

MEDIA	UNTREATED	IRRADIATED	HEATED
	0000 omitted		
Nutrient agar.....	57	20	27
Nutrient agar plus blood.....	57	65	102
Nutrient agar plus glucose.....	60	45	105
Nutrient agar plus yeast.....	61	25	27
Infusion agar.....	61	38	189
Tomato juice milk powder agar.....	54	69	237

TABLE 4

*The viability of bacterial spores before and after lethal exposures to HgCl<sub>2</sub>*

MEDIA	NUMBER OF VIABLE SPORES PER MILLILITER			
	<i>B. cohaerens</i>		<i>B. subtilis</i>	
	Untreated	HgCl <sub>2</sub>	Untreated	HgCl <sub>2</sub>
	0000 omitted		0000 omitted	
Nutrient agar.....	134	10	88	52
Nutrient agar plus blood.....	140	24	123	129
Nutrient agar plus glucose.....	120	1200	218	230
Nutrient agar plus yeast.....	131	43	47	42
Infusion agar.....	142	351	237	230
Tomato juice milk powder agar.....	149	414	232	219

action which in turn depends on the severity and duration of the treatment. This fact is illustrated in table 5. When 50 per cent of the culture survived, the survival ratios closely resembled those obtained with untreated spores. As the mortality is increased, however, these ratios increase rapidly and progressively. If the results with this organism are representative for

other species the effectiveness of enrichment substances in subculture media will depend on the intensity of the killing influence and may be quite limited in the presence of mild killing agents.

In the next experiment, consideration was given to the time at which enrichment was supplied. In this experiment spores of *B. cohaerens* were so heated that direct subculture on glucose agar yielded after incubation a convenient number of colonies, while similar inoculations poured with nutrient agar permitted no ger-

TABLE 5

*The development of irradiated and heated B. cohaerens spores in different media with changes in the proportion of survivors*

MEDIA	PERCENTAGE SURVIVAL							
	50		25		10		1	
	Irradiated	Heated	Irradiated	Heated	Irradiated	Heated	Irradiated	Heated
Nutrient agar . . . . .	111	121	101	37	31	3	10	3
Nutrient agar plus blood . . . . .	147	173	278	128	180	403	130	748
Nutrient agar plus glucose . . . . .	108	185	146	300	99	1000	94	6000
Nutrient agar plus yeast . . . . .	72	104	60	10	21	3	7	1
Infusion agar . . . . .	141	176	174	231	123	500	80	3167
Tomato juice milk powder agar . . . . .	118	177	151	226	49	667	42	2650
Ratio nutrient: glucose agar . . . . .		1:1+		1:8		1:333		1:2000
Ratio nutrient: blood agar . . . . .	1:1+		1:2+		1:5		1:13	

mination. A large series of nutrient agar plates were poured immediately after exposure. As soon as the agar became solid the glucose solution was spread uniformly over the surface of three plates with a sterile applicator. All the plates were then incubated and at desired intervals three plates were treated with the glucose solution as described. All plates containing spreaders were discarded. These were troublesome only when the enrichment was added to the freshly poured plate. The results obtained are presented in table 6. With *B. cohaerens* the results are rather surprising. Within three hours there is a definite



decline in the number of spores able to germinate. This trend increases so rapidly that after 24 hours only an occasional spore is viable. Thereafter glucose was practically without effect as a means of inducing the germination of these spores. With CC and *B. subtilis* little or no loss of viability occurred during the first 24 hours, but from this time on CC spores rather rapidly lost their power to germinate, so that 8 to 10 days after the initial seeding they were practically non-viable. *B. subtilis* spores retained their viability unchanged for the first five days and then declined rapidly. The reason for this rather rapid loss of via-

TABLE 6

*The growth of heated spores with changes in the time at which glucose enrichment is supplied*

TIME AFTER PLATING ENRICHMENT WAS ADDED	<i>B. COHAERENS</i>	<i>B. SUBTILIS</i>	CC
Immediately	206	36	78
3 hours	161	38	82
5 hours		35	79
24 hours	3	36	70
2 days	0	41	47
3 days	0	35	26
4 days	0	50	10
5 days	0	39	6
8 days	0	40	1?
10 days	0	6	0

Suspensions contained approximately 1,000,000 viable spores per milliliter before exposure.

bility of spores seeded on an inadequate medium is not entirely clear. When *B. cohaerens* spores heated as specified above were examined microscopically on nutrient agar blocks, it was observed that a large number of the cells became swollen rather rapidly and lost their characteristic refractive property, but the germination process did not continue visibly beyond this stage.

In view of these observations, the logical deduction would be that heated spores undergoing incomplete germination (because of an inadequate medium) have thereby assumed the characteristics of the vegetative form, and consequently die rather rapidly in the absence of a favorable nutrient environment. This view

is in harmony with some of our unpublished data in which it was observed that susceptibility to heat in germinating spores occurs a considerable time before visible rupture in the spore wall.

#### DISCUSSION

The foregoing observations show clearly that organisms which survive drastic killing factors are more fastidious in their food requirements than the less resistant individuals which predominate in an unexposed portion of the culture. While the reaction is apparently common to all organisms, it is not surprising that different species vary considerably in the degree to which they exhibit it. The advantages of a fermentable carbohydrate in the bacteriological examination of milk were pointed out by Sherman (1916). It is interesting to note that lactose enrichment yielded a higher count with both raw and pasteurized milk, but the percentage increase was much greater with the latter. More recently Ayers and Mudge (1920), Safford and Stark (1935), and others, confirmed this but it is apparent that the broader implications to be drawn from these observations were not considered. Morrison and Rettger (1930) made the significant observation that enrichment substances present in the heating or subculture media entirely eliminated dormancy and skips exhibited by heated spores subcultured in plain nutrient broth. Williams (1936) recently reported the superiority of casein digest as a basic plating medium for the detection of flat-sour spores in sugar. The fact that the sugar solutions were boiled before they were used is of interest in view of the results obtained.

In seeking an explanation for the dissimilar food requirements of resistant and non-resistant spores, two theories suggest themselves neither of which can easily be proved or refuted. The relatively small proportion of resistant spores which make up every culture may by inheritance possess metabolic requirements different from the vast majority. If this were true destructive influences by eliminating all the weak and moderately resistant individuals would serve to concentrate those cells more exacting in their growth requirements and consequently more responsive to growth-promoting substances. This view is not necessarily

incompatible with the findings that different enrichment substances may be required depending on the nature of the destructive action, for proof is lacking that spores which are resistant to one influence are necessarily those resistant to other influences, and there is some evidence to the contrary (Duggar and Hollaender, 1934). The possibility exists, also, that spores surviving destructive influences have sustained some form of injury, impairment of certain enzymic functions perhaps, which has rendered them unable to metabolize nutrients formerly utilizable. Oster (1934) has shown that monochromatic ultra-violet light may cause different degrees of damage with yeast, and that certain metabolic functions of the cells are affected before reproduction is stopped. Rahn and Barnes (1933), however, reported that loss of reproductive ability preceded loss in fermentation ability for the majority of yeast cells exposed to irradiation. The second hypothesis seems somewhat less likely in view of the fact that significant differences in the germination of heated *B. cohaerens* spores in the different media were not noted until more than 50 per cent of the cells were killed. If injury were the cause it is difficult to understand why this should not be reflected in the less resistant half of the culture.

#### SUMMARY

The results of experiments are reported which indicate that bacterial spores which survive drastic killing influences are much more exacting in their nutritive requirements than the less resistant individuals which comprise the bulk of the viable population before treatment. Heat-surviving *Escherichia coli* show the same reaction.

Enrichment substances incorporated in culture media are essential for the accurate enumeration of bacteria previously exposed to highly lethal factors.

The enrichment substances to be used will vary with the species of organism and the nature of the destructive action.

The effectiveness of enrichments in the determination of viability varies with the extent of the killing action. The favorable influence of growth-promoting substances increases as the per-

centage mortality increases and may be negligible in the presence of low mortality.

Spores which survive destructive influences when seeded in inadequate media lose their viability rather rapidly.

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# CELL INCLUSIONS AND THE LIFE CYCLE OF AZOTOBACTER

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The complicated life cycle described by Löhnis and Smith (1916, 1923) for *Azotobacter* has not been fully confirmed by subsequent investigation. Jones (1920) who had previously reported reproduction by gonidia, accepted the doctrine of symplasm but was not able to confirm conjunction, the formation of endospores, or filtrability. Mulvania (1919) confirmed reproduction by budding and gonidia. de Regel (1932) accepted the gonidial method of reproduction and filtrability but rejected conjunction, symplasm, and transmutation of cells. Beauverie (1925) failed to confirm any of the salient features of a cyclogenic life history.

In view of the conflicting results obtained by various workers it appears that a further study is needed. This is especially desirable since most of the recent ideas of bacterial life cycles originated in a study of *Azotobacter*. Similar life cycles have been described for many species of bacteria and there is a tendency to regard bacterial cyclogeny as an established fact.

The present investigation is concerned with the structure and methods of reproduction of *Azotobacter* with special attention to the nature and function of the granular bodies which occur in the cells at certain periods in the life of a culture.

Eight strains of *Azotobacter chroococcum* and one strain of *Azotobacter beijerinckii* have been studied. Two of the strains were freshly isolated from soil. The remainder were supplied by Dr. E. B. Fred from his collection of stock cultures. Before beginning the experiments, each strain was tested to determine purity and conformation to type. It is known that all strains were pure and typical.

Each strain was cultivated on various liquid and solid culture media, and the development was followed by numerous microscopic examinations. The media employed included mannitol nitrate solution and mannitol nitrate agar prepared according to Löhnis and Smith (1916), mannitol soil extract agar, mannitol soil extract, beef extract broth and agar with various sources of additional carbon.

For most purposes examination was made of living material in hanging drop mounts or sealed flat wet mounts in dilute staining solutions. Fixation with formalin and alcohol was superior to heat fixation for the preparation of fixed stained films. Special methods later to be described were employed for staining the granular cell inclusions.

In order to follow the cytomorphic changes cultures were examined at close intervals for a period of 36 hours and at longer intervals as the cultures aged. The oldest material examined was from cultures about six months old. The older culture tubes and flasks had not been previously opened.

#### CELL INCLUSIONS

Granular bodies have been observed in cells of *Azotobacter* by all workers, but many different opinions have been expressed as to the nature and function of these bodies. It is desirable to review this phase of the subject briefly.

Beijerinck (1901) spoke of the granules as fat bodies. Fisher (1906) reported the presence of volutin. Prazmowski (1912) concluded that the cells contain glycogen and a true nucleus which may become dispersed as granules in the cytoplasm. Mencl (1911) regarded the granules as chromidia and the equivalent of a true nucleus. Issatschenko and Giljarowski (1922), Schmidt (1920), and Bonazzi (1915) reported volutin.

Jones (1913) appears to have been the first to assign a reproductive function to the granular bodies. He distinguished two types on the basis of reaction to stains. He regarded the stainable granules as reproductive bodies and designated them as gonidia. The nonstainable granules appeared to be glycogen.

Löhnis and Smith (1916) wrote as follows:

If the granules are very small they are nearly always easily stained. The larger ones on the other hand usually remain entirely unstained when treated with aqueous solutions of aniline dyes. Undoubtedly these granules are of different nature. Some may be fat, glycogen, or other metabolic products. Most of them, however, are living entities.

There is no evidence that microchemical or solubility tests were employed to distinguish the living entities from lifeless inclusions and (throughout the remaining discussion all granular bodies are referred to indiscriminately as gonidia or as nuclear material.)

Stapp (1924) identified the stainable granules as volutin and non-stainable bodies as fat. He reported negative results for glycogen tests. de Regel (1932) maintained that the granular bodies are gonidia since tests for fat, volutin, and glycogen failed to give typical reactions. Zeigenspeck (1930) expressed quite a different view. According to his interpretation the bodies are living units which invade the cells and grow therein as parasites.

In a previous report the writer (1937) called attention to the fact that cells of *Azotobacter* deposit fat bodies and volutin. It is desirable to record here the evidence on which this conclusion was reached.

Tests for fat were carried out according to the naphthol-blue method of Dietrich and Liebermeister (1902) and the various methods described by Eisenberg (1909). The large refractive granules, which fail to stain with aqueous solutions of aniline dyes (fig. 4), stain intensely by the various fat staining methods. There seems no reason to doubt the lipoidal nature of these bodies or to regard them as in any manner different from similar bodies which occur in such well known species as *Bacillus mycoides*, *Bacillus anthracis*, yeasts, and other fungi.

Various functions have been assigned to non-stainable granules which occur in bacterial cells and much confusion has existed in the past. Meyer (1904), Grimme (1902), Eisenberg (1909), Lewis (1934) and others have regarded the bodies as lifeless cell inclusions which function as reserve food. Ruzicka (1907) considered non-stainable material of *B. anthracis* cells as the achromatic portion of a nuclear cell and designated the bodies as



sporoids to indicate their spore-like appearance. Various workers have spoken of such granules as sporogenous granules or prespore material. Schneider (1894) observed non-stainable bodies in bacteroids of rhizobia and identified them as endospores. Löhns and Smith (1916) and de Regle (1932) have spoken of the fat bodies in *Azotobacter* as non-stainable gonidia. A similar view was held by Gibson (1928) concerning the refractive granules of rhizobia. Dietrich and Liebermeister (1902) concluded that the fat bodies of *B. anthracis* act as oxygen carriers.

It can be argued that fat staining reactions and solubility tests serve merely to demonstrate the presence of fat and do not exclude the possibility that substances of a living nature may be present. According to this view the bodies might consist of chromatin granules and cytoplasm embedded in a fatty matrix. So far as the writer knows no such theory has been advocated for fat bodies in other groups of plants. It would seem as logical to apply this interpretation to the bodies in yeasts, fungi, and higher plants.

It is not necessary, however, to rely on analogy to determine the true nature of the bodies in question. There is abundant evidence opposed to the notion that they are living entities of the bacterial cell.

The evidence is conclusive that fat bodies in *B. anthracis*, *B. mycoides* and other species disappear during the late stages of spore formation. Although the vegetative cells contain numerous granules, which do not enter directly into spore formation by fusion, the residual cytoplasm of the sporangium, at maturation of the spore, contains no granules. This has the appearance of digestion and absorption of stored food.

A similar disappearance of granules has been reported by Bonazzi (1921) for cells of *Azotobacter* under experimental conditions. The experiments show that the granular bodies disappear from cells suspended in NaCl solution. He concludes that the bodies function as food when no more sugar is available. His results are in accord with the principle designated by Duclaux as the "phénomène de vie continuée." There seems no good reason

to believe that such autophagy would occur in the case of living units.

Evidence that such bodies are lifeless is found in lack of capacity to germinate as shown by Ruzicka (1907), Cunningham (1931), Stapp and Zycha (1931) and others. Direct evidence by microscopic methods is of doubtful application in the case of *Azotobacter*. Many vegetative coccoid cells in old cultures are reduced to a size of about 0.8 to 1.0 micron in diameter. Such cells frequently contain a single fat body which almost fills the cell. This condition is evident when the cells are stained with a fat-staining method but in the living unstained condition such a cell might be regarded as a free fat body and thus lead to erroneous interpretation (figs. 3 and 4). It appears most probable that the positive germination tests reported by de Regle (1932) were obtained in this manner. Less direct but valuable is the evidence from studies on filtrability. This subject will be discussed in a later paragraph.

When all of the evidence is duly considered we conclude that the refractile granules of *Azotobacter* are lifeless storage products of a fat-like nature which function as reserve food. To accept any other interpretation would be to disregard an abundance of substantial evidence in favor of an unsupported hypothesis.

Tests for volutin have been well known since the pioneer work of Arthur Meyer (1904), and Grimme (1902). It is well known that volutin occurs in many species of bacteria. Some of the granules of *Azobacter* stain intensely with aqueous solutions of aniline dyes. When stained with methylene blue they resist destaining with 1-per-cent sulphuric acid. They are soluble in water heated momentarily to the boiling point or within a few minutes at 85°C. Moreover, they are metachromatic when stained with methylene-blue solution.

Permanent stained preparations may be made by any of the numerous methods proposed for the differential staining of diphtheroids. Very satisfactory results were obtained by staining fixed films with Loeffler's methylene blue, followed by Bismark brown and Lugol's iodine solution. The volutin grains stain in-

tensely by this method and are sharply differentiated from cytoplasm and fat bodies (fig. 3). The most striking differentiation occurs when living cells are stained with methylene blue and treated with 1-per-cent sulphuric acid.

On the basis of these microchemical and solubility tests, we are forced to conclude that the stainable granules of *Azotobacter* are volutin rather than chromidia, or gonidia, and that they are, therefore, not concerned in the reproductive process.

#### CONJUNCTION AND SYMPLASM

According to Löhnis and Smith (1923) all young cultures of *Azotobacter* show cells in a process of conjugation connected by a tube-like structure. Such pairs of cells are numerous in cultures but the appearance is due to incomplected fission. Throughout the earlier phases of growth the cells are borne typically in pairs (figs. 1 and 2).

The most critical stage of the life cycle proposed by Löhnis and Smith is the symplastic phase. According to this theory, bacteria exist in an organized form and in an amorphous condition from which new cells originate by formation of regenerative units.

Examination of material from old cultures by vital staining methods or fixed stained films affords convincing proof that the symplasm consists of a mixture of empty cell membranes, fat and volutin balls, with some cellular elements which have not undergone complete autolysis. The "regenerative units" were probably volutin balls which may show such differences in size as to suggest minute and larger cocci (fig. 3).

One of the cultures freshly isolated from soil was contaminated with an amoeba which lived on the cells of *Azotobacter* and in the precyst stages showed a startling resemblance to some of the descriptions of symplasm. The cysts of this organism were not unlike Löhnis and Smith's 1923 description and illustrations of "macrocyts" which occurred in one of their cultures of *Azotobacter beijerinckii* (plate 9, fig. 66).

Beijerinck (1901) noted *Amoeba* in cultures of *Azotobacter* and devised methods for isolation and maintenance of amoeba cultures. It appears that such mixed cultures may persist in-

definitely. The culture considered here has been under observation for several months during which time numerous subcultures have been prepared. Macroscopically the appearance is that of a normal pure culture, but microscopic examination shows that the protozoan contaminant is still present in undiminished numbers. An uncritical examination of such a culture could have suggested the original idea of symplasm.

The writer believes that the theory of a symplastic stage in the life history of bacteria is erroneous and deserves no further place in bacteriological literature.

#### TRANSMUTATION OF CELLS

According to Löhnis and Smith (1923), *Azotobacter* cells may become transformed into cells of an entirely different type which are cultivable as such, and give rise to strains identical with other well known species. They recognized seven distinct cell types and established fourteen species belonging to five different genera.

We are accustomed to expect more or less extreme polymorphism in the cells of a bacterial culture during the course of its development, but such extreme transmutation must be doubted until fully confirmed.

Examination of cultures during different phases of growth reveals such an orderly transformation as has been shown by Henrici (1928) for *B. megatherium*. Old cultures consist principally of oval and spherical cells containing numerous granules (figs. 3 and 4). Some thick-walled cells, arthrospores, are invariably present (figs. 5 and 6). There is great variability as to size and shape but almost complete uniformity in the presence of granular cell inclusions. Very large involution forms are generally noted although not abundant.

When transfers are made from old cultures to fresh culture media and examined at regular intervals for a period of 24 to 36 hours, the sequence of transformation is readily followed. Very young cultures consist almost entirely of elongated cylindrical cells without fat bodies and not more than one or two grains of volutin. The cell membrane is thin, the cytoplasm is dense and

hyaline and stains intensely with dilute aqueous solutions of aniline dyes. Such cells are more or less actively motile.

In older cultures (12 to 18 hours) the cells have become somewhat shorter and occur principally in pairs. They are generally oval, but typical lancet-shaped diplobacilli are abundant. Fat bodies and several grains of volutin are present in such cells. As the growth rate slows down continued cell division results in the formation of short bacilli which round out to form perfect cocci. The transformations of the bacillary to the coccus form occurs within 24 hours at which time numerous cocci may be noted. The cocci are capable of multiplication as such unless transformed to fresh culture media. Within 36 hours the culture has returned to a mixture of cell types similar to that with which it started.

This behavior involves nothing unusual unless it be the more pronounced tendency to form cocci. The transformation of cells within a culture has been designated by Henrici (1928) as cytomorphosis. He recognized three types of cells, embryonic, mature, and senescent, on the basis of morphological and physiological characteristics. Experience has shown that an orderly transformation occurs in various species when transfers are made from old cultures to fresh media.

Careful search through numerous preparations has failed to reveal endospore formation in any of the strains included. The evidence appears to be conclusive that *Azotobacter* does not form endospores.

Plating from cultures at different periods of growth gave no evidence of such transmutation as was described by Löhnis and Smith. On the other hand, the several strains proved to be remarkably stable.

It is difficult to explain such extreme splitting up into new types as that described by Löhnis and Smith for *Azotobacter*. This is no ordinary case of bacterial dissociation such as we have become accustomed to expect but must be regarded as transmutation to other genera and species.

The writer believes that a partial explanation can be found in the description of original cultures employed by Löhnis and

Smith in their studies. There are reasons to doubt the purity of some strains or even whether they were properly identified at the beginning. Four strains are described as consisting of large sporulating cells only. Two strains consisted of small sporulating rods, three of irregular fungoid cells, four were made up of small non-sporulating rods, one of coccoids and one of dwarfed cells.

Concerning the purity of the cultures they wrote as follows:

Although it was known in only some of the cases where atypical growth occurred that this was not due to contamination, we decided to include all atypical strains in our studies and to base our ultimate decision on the outcome of these experiments.

Their tabulation of transformation from original type of growth shows that in a total of 188 transformations 153 occurred in the atypical strains. Cultures which consisted of large spore-free cells at the beginning gave rise to 35 variant types consisting of white, red and yellow cocci; white and yellow spore-free small rods; white fungoid growth; and sporulating rods both large and small.

Since these types corresponded with the atypical strains and atypical strains produced some typical colonies of *Azotobacter* it was thought logical to conclude that transformations could occur in either direction.

It appears more reasonable to believe that some of the original cultures were contaminated with various species at the beginning of the experiments. These results have not been confirmed by any subsequent investigation. In the light of experience with the eight strains studied here it is believed that confirmation is impossible with pure strains of *Azotobacter*.

#### FILTRABILITY

Attempts to obtain growth from filtrates of cultures were not successful. The cultures were grown in mannitol broth, Ashby medium and on mannitol agar. The agar cultures were washed down with sterile mannitol mineral solution and diluted to 100 cc. After centrifuging, the liquids were passed through Berke-

field N filters and left to incubate in the filter flasks to afford optimum conditions of aeration. Cultures for filtration were 1, 2, and 3 weeks old. The filtrates remained clear during an incubation period of 4 to 6 weeks. Attempts to recover the organism by plating from incubated filtrates gave negative results.

The occurrence of a filtrable stage in the life history of *Azotobacter* has been supported by Löhnis and Smith (1916), Miede (1923) and de Regel (1932), but received no support in the experiments of Jones (1920), Klieneberger (1930) and Roberg (1935). Since the smallest visible true cells are about 0.8 to 1.0 micron in diameter there seems little reason to expect filtrability under properly controlled experimental conditions.

This question has been most thoroughly studied by Roberg (1935), who employed various species of *Azotobacter* and different types of filters. It would appear that his methods were suitable and the experiments sufficiently ample in scope to afford reliable and conclusive results.

#### DISCUSSION

The advocates of bacterial cyclogeny have based their claims on reversion of variants to the original type, and methods of reproduction other than binary fission. These are matters which deal with the most fundamental characteristics of the bacteria. The opponents of cyclogeny have maintained that reversion does not occur in all cases and that reproduction by gonidia, conjugation, symplasm, and heterogamy has not been proved.

Although *Azotobacter* has been regarded as one of the best examples of a complete cyclogenic life history, and has served as a standard of reference for the study of other bacterial species, it has been found impossible to confirm such a life history.

It may be noted that similar life cycles have been described for other species which could not, however, be confirmed. The most striking case is that of the life cycle proposed by Oesterle and Stahl (1930) for *Bacillus mycoides*. Subsequent investigation by Stapp and Zycha (1931), den Dooren de Jong (1933), and Lewis (1932) failed to support their conclusions. The report of Cunningham and Jenkins (1927) on *Bacillus amylobacter* was not

confirmed by Bucksteeg (1935). Holman and Carson (1935) concluded that streptococci are not transformed to spore-bearing rods as claimed by Evans and that her results were due to faulty methods. Numerous workers have failed to confirm the occurrence of a filtrable phase in well known species. Clauberg (1928) concluded that the Almquist granules of *Eberthella typhi* are not reproductive bodies.

In the light of available evidence it appears probable that the life history of most bacteria is extremely simple, consisting of a repetition of cell division.

#### SUMMARY

The stainable granules which develop in the cells of *Azotobacter* consist of volutin. The non-stainable granules are fat bodies. Neither gonidia nor visible chromidia occur. Appearances resembling conjugation are due to incompleated fission. The so-called symplasm consists of gum, cell walls, fat bodies, volutin balls, and cellular elements.

Transmutation of cells to a different type cultivable as such to form culture phases different from the original does not occur in any of the several strains tested. Filtrability tests gave negative results.

*Azotobacter* reproduces by binary fission, and by arthrospores. The life history is relatively very simple.

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## PLATE 1

FIG. 1. *Azotobacter chroococcum* from an 18-hour culture on Ashby's agar, showing incomplete fission of diplo-bacilli and cocci.

FIG. 2. *Azotobacter beijerinckii*. Same as above.

FIG. 3. *Azotobacter chroococcum* from culture 48 hours on beef extract peptone sucrose agar. Stained with Loeffler's methylene blue, bismark brown and Lugol's iodine solution. The cells contain volutin grains.

FIG. 4. *Azotobacter beijerinckii* from culture 5 days old on Ashby's mannitol agar showing fat bodies and volutin. Stained with methylene blue and Lugol's solution.

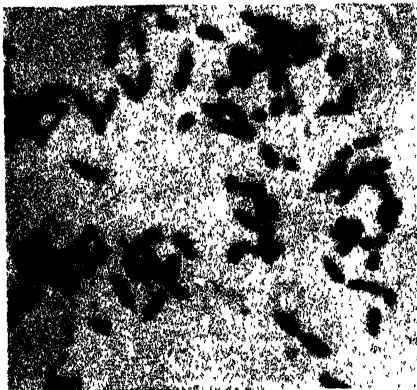
FIG. 5. *Azotobacter chroococcum* from culture 10 days old on beef extract peptone agar showing thick walled arthrospores.

FIG. 6. *Azotobacter beijerinckii*. Same as figure 5.

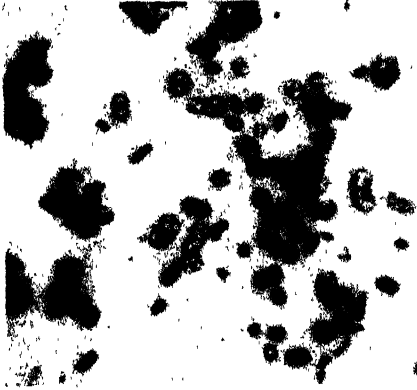


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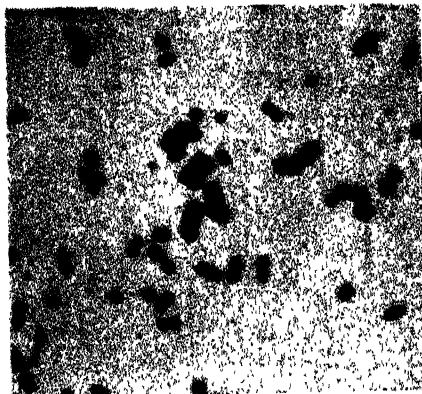
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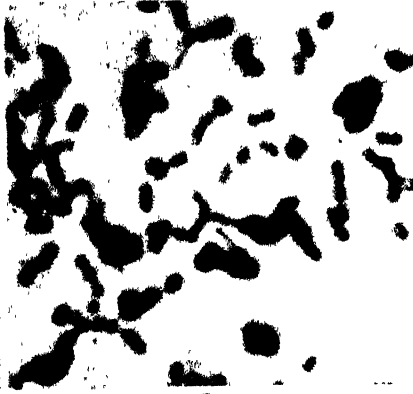
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# THE OXIDATION OF SEWAGE BY BACTERIA IN PURE CULTURE

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The activated-sludge process of sewage treatment, essentially a biological process, purifies sewage at rates which exceed very greatly those observed in ordinary biochemical oxidation. The natural assumption has been that there is a rapid adsorption of the dissolved and colloidal material by the sludge and that this adsorbed material is then biochemically oxidized at the ordinary rate.

In our studies of the activated sludge process a large number of microscopical examinations of activated sludge were made covering samples from plant scale units in other cities, from the local experimental unit, and from small laboratory set-ups. Regardless of the source of the sludge, if it were a good sludge, it was found that the predominating components of the flocs were bacteria. These bacteria were packed together usually in solid masses, sometimes with finger-like or pyramidal branches extending from the mass. These bacterial masses or flocs, held together in a gelatinous matrix, which appears to be a gummy exudate of the bacterial cell, may be likened to huge bacterial colonies suspended in a liquid medium.

To obtain information regarding the function of these bacteria in the activated sludge process masses representing the predominant variety of floc were picked out with capillary pipettes and washed through a series of changes in sterile water. During this washing process the floc was teased with fine tipped needles of glass and as much as possible of adherent material, detritus, etc., separated from it. In this washed condition the floc not only

appeared to be composed entirely of bacterial cells packed closely together but also all of the bacterial cells appeared to be of the same type. After the washing the remaining bacteria in the floc were dispersed in a small amount of water and planted. Organisms isolated which produced floc were repurified until pure culture growth was assured. Three different strains of these bacteria with the same general characteristics have been isolated and studied in pure culture. One of these cultures was isolated from activated sludge from the station experimental plant while the other two were obtained from two separate samples of activated sludge received from the Lancaster, Pa., plant.

In a preliminary report, Butterfield (1935), a detailed description of the morphological, cultural and physiological characteristics of one of these strains of bacteria was presented. In addition it was shown that this bacterium in pure culture, both in synthetic sewage and in sterilized natural sewage, produced a sludge floc which simulated activated sludge to a remarkable degree. This pure-culture "activated" sludge removed, during a 3-hour aeration period, from 41 to 84 per cent of the oxidizable material present in polluted water or sewage. This removal of pollutional material from the supernatant or effluent represented an over-all or total purification. The material may have been oxidized biochemically by the massed bacteria, it may have been adsorbed by the sludge, some of the material may have been utilized for the synthesis of new sludge solids, or each of these processes may have played a part in the purification accomplished.

While the development of sludge in sewage under aeration has been universally observed, the source of this sludge has been to a great extent a matter of conjecture. The source of sludge developed from natural sewage might be the solids originally present as altered by aeration and physical changes, augmented by the precipitation of dissolved and colloidal constituents. To be able to observe clearly the influence of pure cultures in sludge production and in the oxidation of material by such sludge it seemed pertinent, in at least a few tests, to permit the bacteria to develop in solutions containing essentially the same dissolved constituents as sewage but which were entirely free of undissolved substances.

Under such conditions sludge production could be readily observed and could be attributed definitely to the bacteria present in pure culture. A synthetic sewage of such characteristics, used in certain experiments, contained: Peptone, Difco, 0.3 gram, Meat extract Liebigs, 0.2 gram, Urea, 0.05 gram, Disodium hydrogen phosphate, 0.05 gram, Sodium chloride, 0.015 gram, Potassium chloride, 0.007 gram, Calcium chloride, 0.007 gram, Magnesium sulphate, 0.005 gram, and distilled water, 1000 ml. Sterilization was accomplished by steam pressure, 15 lbs. for 15 minutes. The mineral salt and the urea content of this solution was approximately the same as that of sewage basing the calculation for urea on the average amount of urea excreted per capita per day and assuming the average daily pumpage of water per capita as the normal dilution. The peptone and meat extract in the solution made its biochemical oxygen demand similar to that of a strong domestic sewage.

The pure cultures developed a well organized floc in this synthetic sewage in from 24 to 48 hours. (In the preliminary publication it was reported that some variety of structural material, cotton fibers, etc., was essential to floc formation; it was, however, found now that such material was not necessary if the rate of aeration was limited to two cubic feet or less of air per hour.) This floc would continue to accumulate under the conditions of the tests until a well developed sludge was present. Microscopic examination of the flocs, of which this sludge was composed, revealed that they were composed principally, if not entirely, of closely packed masses of bacteria cells surrounded by a gelatinous matrix. This progressive production of a sludge, which simulated natural activated sludge to a marked degree, by these organisms in pure culture, especially in synthetic sewage free from undissolved particles, is remarkable and the purification accomplished by each of the sludges under aeration is a definite indication of their significance in the activated sludge process.

The present report will give the results indicating the portion of this total purification which, after various periods of aeration, may be attributed to biochemical oxidation alone.

In preparing pure culture sludges for these studies the three



strains of bacteria referred to above were employed, five sludges were developed on sterilized natural sewage and two on sterile synthetic sewage. The pure culture sludges studied were developed under aseptic conditions by the fill-and-draw method frequently employed for the development of activated sludge.<sup>1</sup> When a sufficient quantity of pure culture sludge had been developed aeration was continued for 24 hours from the time of the last change. Thus the supernatant with the sludge had been subjected to the action of the sludge for 24 hours. The sludge plus supernatant was then thoroughly mixed and two duplicate portions of exactly one liter each were drawn off into sterile cylinders designated as A and B. The sludge in the two cylinders was allowed to settle for 30 minutes. The amount of supernatant that could be withdrawn by a sterile siphon without disturbing the settled sludge was removed. While the amount thus withdrawn varied in different experiments, exactly the same quantity was removed from each member of each pair of cylinders concerned in one test and the amount accurately determined. In the case of cylinder A this quantity was replaced with sterile sewage (natural or synthetic) and in cylinder B with oxygen-demand-free dilution water. These mixtures A and B were transferred to aeration bottles of four-liter capacity, designated similarly A and B, which were clamped to a closed system aeration apparatus. The apparatus and procedure employed was a modification of that described by Theriault and McNamee (1931) for the determination of oxygen requirements by the aeration method. Aeration was started at once and oxygen determinations were made initially and after various periods, usually after  $\frac{1}{2}$ ,  $1\frac{1}{2}$ , 3, 5, 10 and 24 hours on both A and B.

At the same time samples of the supernatant and of the sterile sewage added were put up for biochemical oxygen demand determinations by the excess-oxygen dilution method. These observations were made after 2, 5, 7, 10 and in some instances

<sup>1</sup> A detailed description of the production of the sludge, of the various procedures involved, of the various factors influencing purification efficiency, and an interpretation of the results obtained as applied to activated sludge plant operation will be presented in the Sewage Works Journal.

15 and 20 days of incubation. As the supernatant contained a pure culture of bacteria and the sewage added was sterile, both of these samples when diluted were inoculated with unsterilized stale sewage to insure a uniform seeding.

For convenience in expressing the results, let

$x$  = the pure culture sludge in one liter of mixed supernatant in terms of suspended solids, assuming that the independent volume of  $x$  is negligibly small.

$y$  = one liter of supernatant,

$z$  = one liter of fresh sterile sewage,

$d$  = one liter of dilution water,

$p$  = fraction of  $y$  used, and

$q$  = fraction of  $z$  or  $d$  used.

Then the fed aeration mixture, A, may be represented by,

$$x + py + qz,$$

and the control aeration mixture, B, by

$$x + py + qd.$$

The oxygen requirements of the control aeration mixtures for various time intervals in eight different set-ups are presented in table 1. In addition, the amounts of massed bacterial growth (sludge) in terms of parts per million of suspended solids, which had been developed at the time of the test, and the 5-day oxygen demand of the supernatant,  $y$ , are given. In table 2 the corresponding oxygen requirements for the fed aeration mixtures for the same experiments are shown. This table contains in addition the 5-day biochemical oxygen demand of the sewage added,  $z$ , as determined by the excess-oxygen dilution method.

As  $x + py$  are equal in each mixture and the  $qd$  had no biochemical oxygen demand, then for a given time interval the difference between the oxygen requirements of the fed mixture and the control mixture, that is, A minus B, is produced by the increment of sterile sewage added or  $qz$ . This method of deducting the oxidation observed in the control mixture from that observed in the fed mixture in order to determine the increase in

the oxygen requirement brought about by the added sewage,  $qz$  has been used in all experiments. In one experiment (No. 3) two controls were made for one feed mixture. In the first control (3b) the pure culture sludge was suspended in 0.09 liter of supernatant and 0.91 liter of dilution water. In the second control (3a) the same quantity of sludge was suspended in 1.0 liter of supernatant. The results show that this variation in the control does not materially alter the additional oxidation observed.

TABLE 1

*Oxidation in control aeration mixtures*

Oxygen used when pure culture sludge suspensions in synthetic or sterile sewage substrate is aerated but not fed

$x$  = pure culture sludge.  $y$  = 1-day old supernatant.  $d$  = dilution water.

EXPERIMENT NUMBER	CULTURE	P.P.M. SUSPENDED SOLIDS = $x$	P.P.M. 5-DAY B.O.D. OF $y$	COMPOSITION OF AERATION MIXTURE	MILLIGRAMS $O_2$ USED PER LITER IN INDICATED TIME IN HOURS					
					†	1†	3	5	10	24
				liters						
1	Z-4	1420	17.6	$x + 0.375y + 0.625d$	7.6	8.9	13.2	17.8		39.1†
2	Z-4	1632	16.2	$x + 0.375y + 0.625d$	6.1	6.9	11.3	18.7		52.1†
3b	Z-1	773	13.8	$x + 0.09y + 0.91d$	7.7	10.1*		15.9†		23.2
3a	Z-1	843	13.8	$x + 1.0y$	11.3	15.8*		20.7†		35.8
4	Z-9	2644	14.5	$x + 0.1y + 0.9d$	5.3	9.9	13.5	32.2	35.9	48.1
5	Z-9	1560	15.0	$x + 0.1y + 0.9d$	12.0	12.8	14.8	18.5	25.2	38.6
6	Z-9	2428	10.6	$x + 0.1y + 0.9d$	8.0	11.7	17.4	19.6	37.0	60.8
7	Z-9	1632	11.4	$x + 0.1y + 0.9d$	9.2	5.1	17.1	15.2	22.9	34.4

\* 2 hours. † 4 hours. ‡ 22 hours.

While this increased oxygen requirement of the fed mixture can be attributed definitely to the addition of the sewage, it cannot be ascribed to the oxidation of the sewage alone without further consideration. Unfortunately the oxygen requirement of the component  $x + py$  of the mixture,  $x + py + qz$ , (A), cannot be determined separately when it is in the mixture. Without such a determination three assumptions may be made in regard to the oxygen requirement of the component,  $x + py$ , of the mixture A, in the presence of added sewage,  $qz$ , as compared

TABLE 2  
*Oxidation in feed aeration mixtures*

Oxygen used when pure culture sludge suspension in synthetic or sterile sewage substrate is fed immediately before experiment and aerated

$z$  = pure culture sludge.  $y$  = 1-day old supernatant substrate.  $z$  = sewage added.

EX- PER- IMENT NUM- BER	CULTURE	F.P.M. SUB- STRATE PERFED = $z$	F.P.M. 5-DAY B.O.D. O.P.E.	SEWAGE ADDED :	COMPOSITION OF AERATION MIXTURE	MILLIGRAMS O <sub>2</sub> USED PER LITER IN INDICATED TIME IN HOURS					
						‡	1‡	3	5	10	24
1	Z-4	1420	287	Sterile sewage	<i>idlers</i> $z + 0.375y + 0.625z$ $z + 0.375y + 0.625z$ $z + 0.09y + 0.91z$ $z + 0.1y + 0.9z$ $z + 0.1y + 0.9z$ $z + 0.1y + 0.9z$ $z + 0.1y + 0.9z$	32.4	56.4	77.0	93.1		151.0†
2	Z-4	1632	345	Synthetic sewage		37.1	86.2	118.2	139.7		210.4†
3	Z-1	877	220	Sterile sewage		28.4	64.5*		91.4†		182.4
4	Z-9	2868	142	Sterile sewage		26.6	50.6	70.2	84.2	111.7	156.0
5	Z-9	1728	170	Synthetic sewage		34.7	69.6	92.6	113.0	131.0	153.9
6	Z-9	2544	170	Synthetic sewage		20.3	50.0	80.4	103.4	136.5	216.6
7	Z-9	1632	142	Sterile sewage		29.6	46.0	63.4	79.6	97.7	138.3

\* 2 hours. ‡ 4 hours. † 22 hours.

with its oxygen requirement in mixture B: (1) That the oxygen requirement of  $x + py$  is increased and its satisfaction is accelerated by the presence of  $qz$  in A; (2) That the oxygen requirement of  $x + py$  is decreased and its satisfaction is retarded by the presence of  $qz$  in A; or (3) That the oxygen requirement of  $x + py$  is the same in A as it is in B and is satisfied at the same rate in both mixtures.

In considering assumption (1) if the oxygen requirements of  $x + py$  were higher in A than they were in B, then in accordance with known laws, it would follow that  $x$  or  $x + py$  would decrease. As a matter of fact in a large series of observations  $x$  or  $x + py$  has never decreased but has always increased. Hence there could be no increased oxidation of  $x + py$ .

With regard to assumption (2) it would appear reasonable to assume that the presence of  $qz$ , (a fresh food material with readily available constituents), with  $x + py$  might have a temporary sparing action on the oxidation of the  $x + py$  component which had been worked over previously by the bacteria, the oxidizing agents, for 24 hours. Such an effect if it actually occurred would tend to decrease the real difference between the oxygen requirements of A and B, or to decrease the indicated oxygen requirement of the added sewage,  $qz$ , which had been satisfied. Thus, this assumption, if true, would act as a safety factor for the deductions which may be made regarding the accelerated oxidation produced by the pure culture sludges.

Considered from another angle, the pure culture sludges (with the exception of experiments 6 and 7 which will be discussed later), had been subjected daily to the same treatment which was given in the tests. This is particularly true for experiments 4 and 5 where the added sewage,  $qz$ , a synthetic medium, could be, and was exactly reproduced from day to day. Accordingly it is reasonable to presume that the residual  $x + py$  at the end of any test period approximated quite closely the  $x + py$  present at the start of such test.

Consequently, as assumption (1) cannot be true, and assumption (2) if true would decrease the magnitude of the results and serve as a safety factor for the conclusion reached, it appears

TABLE 3  
Oxidation of added increment  
Oxygen used to oxidize the increment of sewage added (milligrams O<sub>2</sub> used by feed culture minus milligrams O<sub>2</sub> used by the control)

EXPERIMENT NUMBER	FEED MIXTURE 1	CONTROL MIXTURE 2*	ADDED INCREMENT (1) - (2)	5-DAY B.O.D. OF ADDED INCRE- MENT	MILLIGRAMS O <sub>2</sub> USED IN OXIDIZING ADDED INCREMENT IN INDICATED TIME—HOURS					
					1‡	1½	3	5	10	24
1	liters z + 0.375y + 0.625z	liters z + 0.375y	liters 0.625z	179.7	24.8	47.5	63.8	75.3		111.9§
2	z + 0.375y + 0.625z	z + 0.375y	0.625z	215.6	31.0	79.3	106.9	121.0		158.3§
3a	z + 0.06y + 0.91z	z + 1.0y	-0.91y + 0.91z	169.5	17.1	48.7†		70.7†		146.7
3b	z + 0.09y + 0.91z	z + 0.09y	0.91z	200.0	20.7	54.4†		75.5†		159.3
4	z + 0.1y + 0.9z	z + 0.1y	0.9z	127.8	21.3	40.7	56.7	52.0	75.8	107.9
5	z + 0.1y + 0.9z	z + 0.1y	0.9z	152.6	22.7	56.8	77.8	94.5	105.8	115.3
6	z + 0.1y + 0.9z	z + 0.1y	0.9z	152.6	12.3	38.3	63.0	83.8	99.5	155.8
7	z + 0.1y + 0.9z	z + 0.1y	0.9z	127.8	20.4	40.9	46.3	64.4	74.8	103.9

z = pure culture sludge. y = 1-day old supernatant. z = sewage added.

\* The necessary quantities of dilution water that were added to make the controls up to one liter have been omitted from this column because they do not enter into the calculation.

‡ 2 hours. † 4 hours. § 22 hours.

logical and safe to assume in interpreting the results of these tests that the difference between the oxygen requirements of the fed aeration mixture A, and of the control aeration mixture, B, represents substantially for the given time intervals the oxygen required for the oxidation of the added sewage,  $qz$ , as effected by the pure culture bacterial mass, (activated sludge), under aeration.

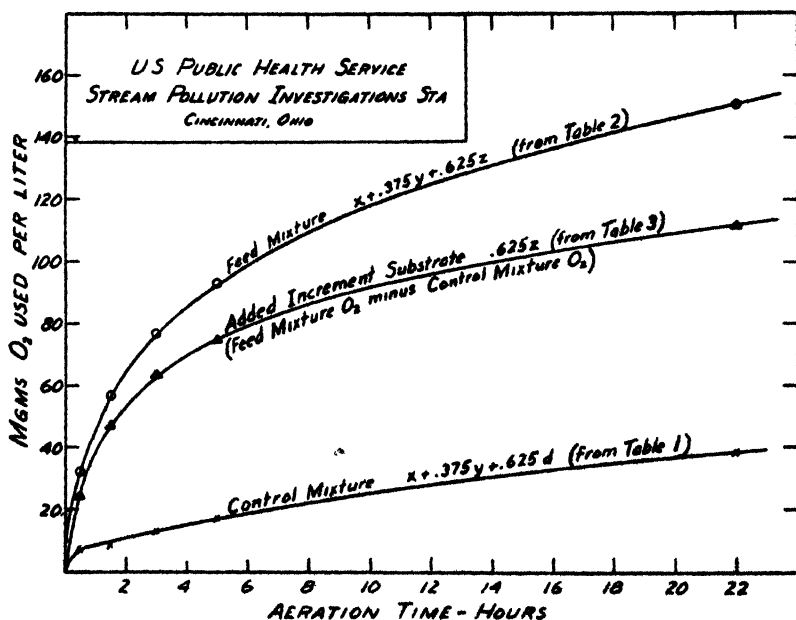


FIG. 1. OXIDATION IN PURE CULTURE SLUDGE-SUBSTRATE AERATION MIXTURES. EXPERIMENT 1

In table 3 the results showing the oxygen requirements of the added sewage for each experiment are recorded. These results were obtained by deducting the values given in table 1 for the oxygen requirements of the control aeration mixtures for the various time intervals from the corresponding values given in table 2 for the oxygen requirements of the fed aeration mixtures for the same time intervals. In experiment 3 two control aeration mixtures were used.

The marked efficiency of the massed cultures (sludge) under

aeration in the oxidation of the added sewage is observed at once. For instance at the 5-hour aeration period in the various experiments 41.3, 56.1, 41.7, 37.7, 40.7, 61.9, 54.9 and 50.4 (average 48.1) per cent respectively of the 5-day biochemical oxygen demand of the added sewage had been oxidized. The significance of this rapid rate of oxidation of the added sewage by these massed cultures can be more readily visualized by referring to figure 1 where the results for a typical experiment (No. 1) are graphically presented. This rapid oxidation is the more remarkable when one considers that it is carried on by bacteria in pure culture undoubtedly more restricted in their food habits than a mixed culture would be. While definite information in regard to the food habits of these bacteria has not been obtained it is known that these organisms do not oxidize nitrogen compounds to nitrites or nitrates.

Attention is directed to the results from experiments 5 and 7 where sludges of 1560 and 1632 p.p.m. as suspended solids were developed on synthetic media. The development of this pure-culture activated sludge in a synthetic sewage with all components in true solution and originally free from suspended particles may be attributed to the pure cultures of bacteria which were introduced. Microscopic examinations of the individual flocs making up these sludges showed them to be composed of closely packed masses of bacterial cells.

To investigate the possibility that these organisms may have been adapted to the rapid oxidation of certain substrates by continued development on the same substrate, in experiments 5 and 7, sludges developed on synthetic sewage were fed in experiment 5 with synthetic sewage and in experiment 7 with sterilized domestic sewage. Similarly in experiments 4 and 6 sludges developed on sterilized domestic sewage, were fed in experiment 4 with sterilized domestic sewage and in experiment 6 with synthetic sewage. The same high rate of oxidation was observed in all four experiments. The average oxidation for the several time intervals was somewhat higher for the sludges fed with synthetic sewage. However, this was true for the sludge developed on sterile domestic sewage as well as for the one developed



on synthetic sewage. This slightly increased oxidation with synthetic sewage probably was to be expected using a substrate composed entirely of dissolved materials readily available for bacterial use.

As Koch's postulates, as they would be applied to such processes, have been fulfilled it is desired to emphasize the primary importance of bacteria in the activated sludge process. These bacteria isolated from activated sludge, reproduced sludge composed entirely of their own cells and cellular products, which simulated activated sludge in appearance and in oxidation efficiency. However, it is not desired to create the impression that these organisms, whose activities are reported, are the only ones which can promote rapid oxidation under such conditions. A number of other bacteria have been tried and found satisfactory. No doubt there is a considerable group of organisms capable of such action. The primary prerequisite for this type of organism, in addition to oxidizing capacity, appears to be the ability to grow in a liquid medium in a massed floc or colony which binds itself together tenaciously enough to remain intact under the agitation of the aeration required to maintain aerobic conditions in the presence of the rapid oxidation taking place.

#### SUMMARY

Organisms which appear to make up the major portion of the bacterial flora of activated sludge have been isolated in pure culture.

Employing these bacteria in pure-culture "activated sludges" have been developed in sterilized natural sewage and in sterile synthetic media whose components were in true solution.

These pure-culture "activated sludges" have been shown to produce a high rate of oxidation of the pollutional material in sewages (synthetic and natural) oxidizing about 50 per cent of the 5-day biochemical oxygen demand in a 5-hour aeration period and about 80 per cent in a 24-hour interval. Nitrogenous materials are not included in this oxidation as these bacteria were not capable of such action.

The production of activated sludge by these bacteria and the

oxidation effected by this sludge definitely indicate the rôle of these bacteria in the activated sludge process of sewage purification.

Appreciation is expressed to the members of the staff of the Station for their assistance, especially to C. C. Ruchhoft, P. D. McNamee and Elsie Wattie who had a major share in the work involved.

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# THE CARBON METABOLISM OF THE CROWN-GALL AND HAIRY-ROOT ORGANISMS<sup>1</sup>

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## INTRODUCTION

The isolation and quantitative determination of certain metabolic products formed from carbon compounds by crown-gall and related bacteria have been studied as part of a larger program (Riker and Berge, 1935) on atypical and pathological multiplication of cells. The general question of the metabolism of these microorganisms has been reviewed in a previous paper (Conner, Peterson and Riker, 1937) dealing specifically with the nitrogen metabolism.

The crown-gall and related organisms do not produce carbon dioxide from sugars rapidly enough to be classed in the conventional manner as gas formers (Smith, *et al.*, 1911; Riker *et al.* 1930; and Pinckard, 1935). Studies on single-cell cultures by Sagen *et al.* (1934), Wilson (1935) and others have shown that while the hairy-root organism produces some acid, the crown-gall organism produces little or no acid. It seems likely, therefore, that substances other than carbon dioxide and acids are produced in considerable quantities. The present paper reports studies on the carbon metabolism of these organisms with special reference to certain products resulting from their action on glucose.

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## EXPERIMENTAL WORK

*Cultures and media.* The crown-gall and hairy-root cultures A-1 and C-1 respectively were progenies of single cells isolated by Wright *et al.* (1930). The identities of the cultures were checked at frequent intervals during the course of the investigation by inoculation of suitable host plants.

After some preliminary experiments to determine the effect of various concentrations of yeast infusion and phosphates, a medium was selected which gave good utilization of the sugar. It had the following composition: 20 per cent press-yeast infusion, 50 cc.; calcium sulfate ( $\text{CaSO}_4$ ), 0.1 gram; magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 0.2 gram; sodium chloride ( $\text{NaCl}$ ), 0.2 gram; sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), 2.9 grams; potassium phosphate ( $\text{K}_2\text{HPO}_4$ ), 8.8 grams; glucose, 10 to 40 grams; and distilled water to make 1000 cc. A solid medium was prepared by adding 15 grams of agar. Any variations from the above amounts are noted later.

*Methods of analysis.* Sugar was determined by the method of Shaffer and Hartman as modified by Stiles, Peterson, and Fred (1926). Carbon dioxide was removed from the cultures by a stream of sterile, carbon-dioxide-free air, absorbed by ascarite, and determined by direct weighing. Bacterial cells and gum were precipitated by alcohol or barium hydroxide, and removed by centrifuging. Other metabolic products were precipitated by a technique described later. Total carbon of the various fractions was determined by the wet combustion method (Heck, 1929).

## RATE OF FERMENTATION

Rates of fermentation of glucose by the two organisms on agar media containing 1, 2 and 4 per cent of the sugar were determined. Ten cubic centimeters portions of each medium were placed in a number of tubes, with special precautions in sloping so as to insure a surface of uniform area in all tubes. Determinations of glucose were made on the combined contents of three tubes every fifth day and the percentage of glucose fermented was calculated. The results of these determinations are

given in figure 1. The hairy-root organism fermented glucose at an appreciably faster rate than the crown-gall organism at all concentrations of glucose studied. Although the percentage of glucose destroyed by either organism was less at the highest concentration, the actual weight of sugar fermented was equal to or greater than that at the lower concentrations.

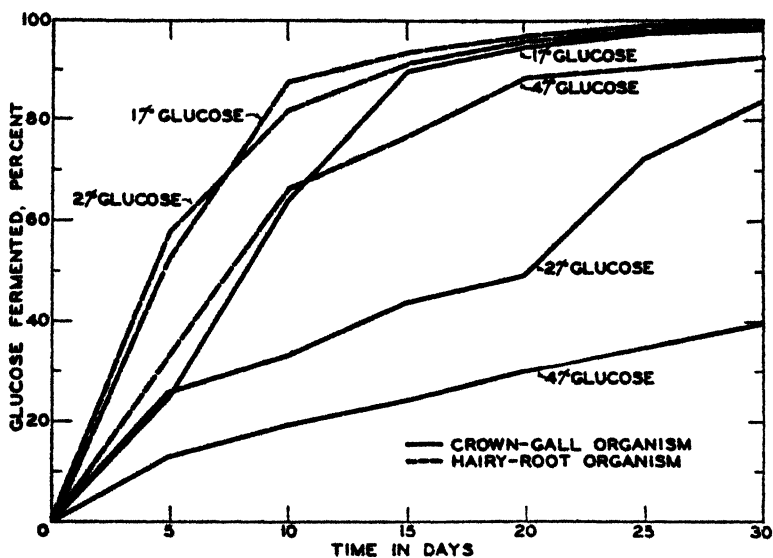


FIG. 1. FERMENTATION OF GLUCOSE BY THE HAIRY-ROOT AND CROWN-GALL ORGANISMS ON AGAR MEDIA

#### MEASUREMENT OF CARBON DIOXIDE PRODUCTION

The set up of fermentations in which carbon dioxide was determined is shown in figure 2. Carbon dioxide production was determined daily by weighing the absorption tube. These results are summarized in figures 3 to 6 inclusive. The scales used in figures 5 and 6 are one-fifth and one-tenth, respectively, as large as the scales used in figures 3 and 4. The results given in each figure are representative of 2 to 6 trials.

The curves show: (1) that the hairy-root organism produced approximately ten times as much carbon dioxide as the crown-gall organism; (2) that the addition of calcium carbonate to an agar

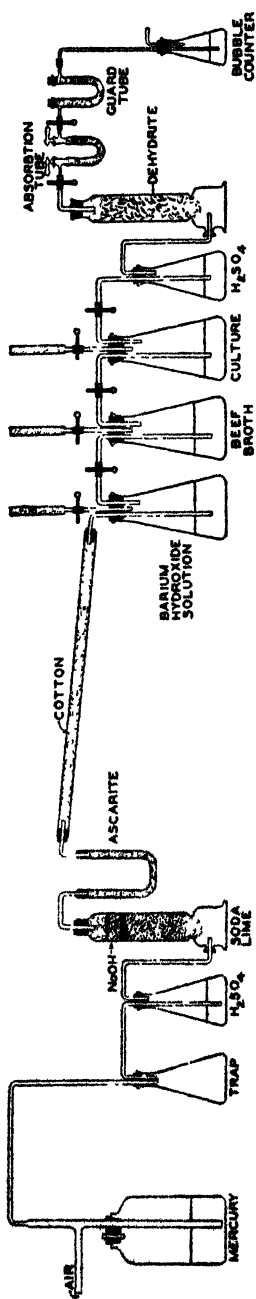


FIG. 2. APPARATUS FOR MEASURING CARBON DIOXIDE PRODUCTION BY CROWN-GALL AND HAIRY-ROOT BACTERIA IN AERATED CULTURES

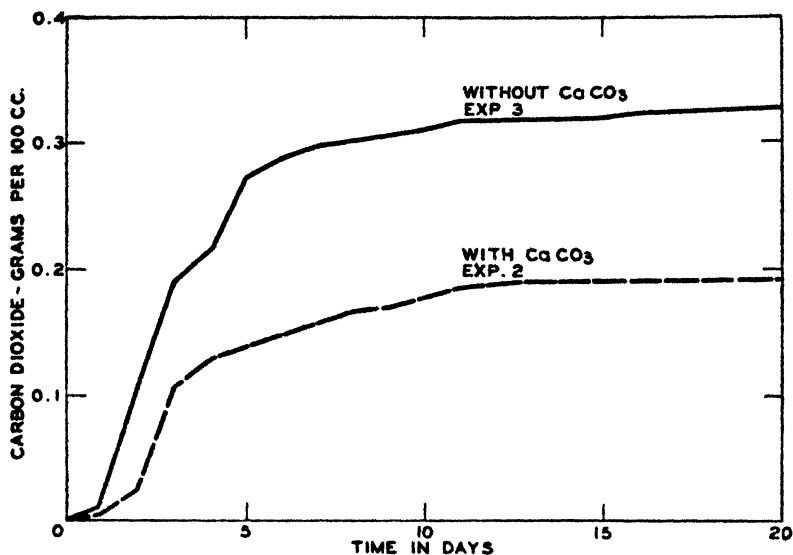


FIG. 3. CARBON DIOXIDE PRODUCTION OF THE HAIRY-ROOT ORGANISM ON AGAR MEDIA

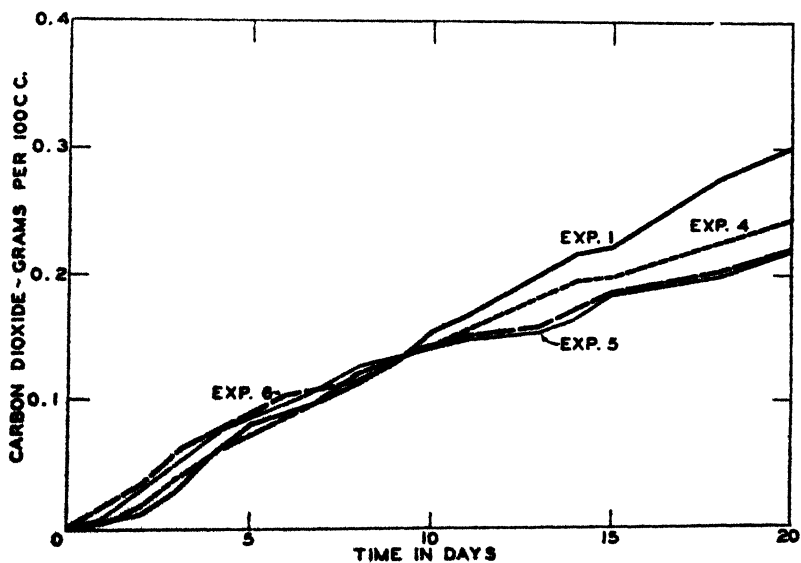


FIG. 4. CARBON DIOXIDE PRODUCTION OF THE HAIRY-ROOT ORGANISM IN LIQUID MEDIA



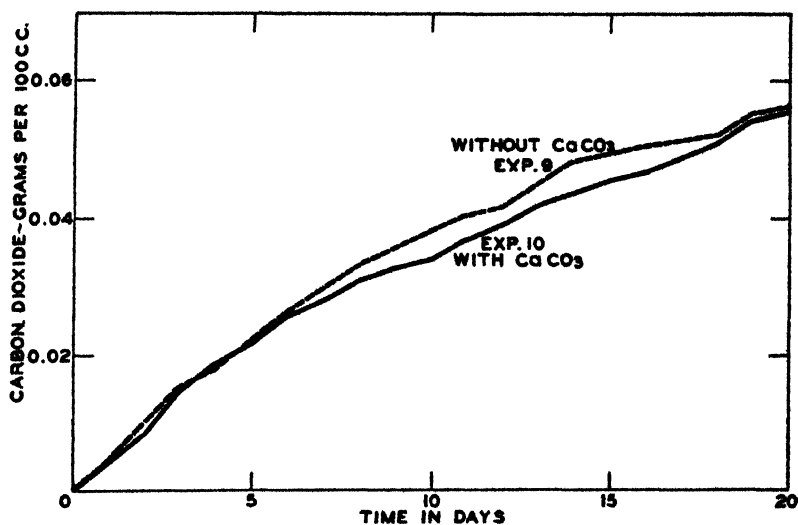


FIG. 5. CARBON DIOXIDE PRODUCTION OF THE CROWN-GALL ORGANISM ON AGAR MEDIA

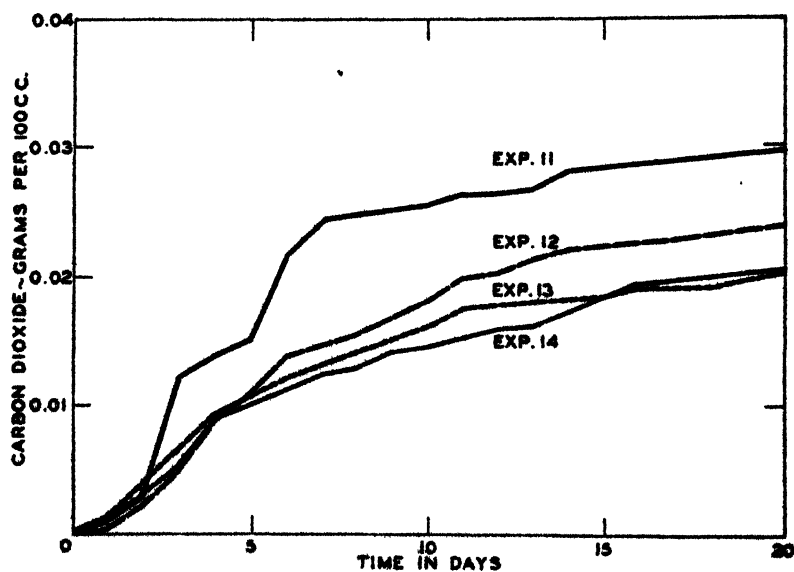


FIG. 6. CARBON DIOXIDE PRODUCTION OF THE CROWN-GALL ORGANISM IN LIQUID MEDIA

medium markedly lowered the carbon-dioxide production of the hairy-root organism, but had no effect on that of the crown-gall organism. The decreased production might be explained in several ways, e.g., less glucose might have been fermented, or less glucose might have been converted to carbon dioxide in the carbonate medium. Other experiments showed that carbon dioxide production did not bear a constant ratio to glucose fermented.

#### IDENTIFICATION OF THE ACIDS PRODUCED BY THE HAIRY-ROOT ORGANISM

The hairy-root organism produces small amounts of acid (Riker *et al.* (1930)) when grown in media containing glucose. The acid was identified by the following procedure. The culture was grown in the basal medium containing 3 per cent glucose. Two liters of medium were placed in three-liter flasks and incubated for at least sixty days in order to have sufficient acid for identification. At the end of this time the cultures were filtered, neutralized, concentrated under reduced pressure, acidified with  $H_2SO_4$  and extracted for 48 hours with ether. Fifty cubic centimeters of water were added to the extract, the ether was removed, and the aqueous solution decolorized with charcoal and filtered. On distilling the filtrate with steam, both distillate and residue were found to contain acid. The distillate was purified by distilling with steam a second time. Only small quantities of acid were obtained, about 4 to 6 cc. of N/10 per liter of culture.

A Duclaux determination on the volatile acid indicated acetic acid. Additional evidence of the identity of this acid was obtained by the preparation of its p-toluidide. For this purpose 50 liters of culture medium were extracted as described, and the volatile acid was converted to its sodium salt. Then 0.4 gram of the sodium salt, 0.4 cc. of concentrated hydrochloric acid and 1 gram of para-toluidine hydrochloride were intimately mixed in a tube and gently heated for 30 minutes. The reaction mixture was cooled, extracted with 95 per cent alcohol and the alcohol poured into 50 cc. of water. The aqueous mixture was evaporated to a volume of about 10 cc., filtered and allowed to cool. The crystals which separated were recrystallized twice from water

and dried. Their melting point was 146°C. uncorrected. A sample of acet-p-toluidide prepared from acetic acid also melted at 146°C. uncorrected. A mixture of the two showed no depression of the melting point.

The residue from the steam distillation was concentrated and used in a number of qualitative tests for the identification of the non-volatile acid. The solution reduced ammoniacal silver nitrate and alkaline potassium permanganate at room temperature. With iodine and sodium hydroxide a precipitate of iodoform was produced in the cold, indicating the presence of a methyl ketonic group in the molecule. Since the tests indicated the presence of a keto acid, the 2-4 dinitrophenylhydrazone of the acid was prepared. After two crystallizations from ethyl acetate the hydrazone melted at 215°C. uncorrected. Since pyruvic acid gives all these reactions the 2-4 dinitrophenylhydrazone of this compound was prepared. This hydrazone also melted at 215°C. uncorrected and a mixture of the two hydrazones showed no depression of the melting point.

#### CARBON BALANCE

A comparison of the carbon in the glucose fermented with that in the known products showed that both organisms must produce other products in considerable quantities. These substances were not volatile, since the sulfuric acid through which the air passed after leaving the culture contained no organic compounds as was demonstrated by analysis for carbon.

A carbon balance was made to show what percentage of the glucose was accounted for by products. The cultures used in the studies of carbon dioxide production, were also used for the determination of (1) reducing sugars, (2) total carbon, and (3) carbon in cells and gum. The cells and gum were precipitated by alcohol. The results of four representative determinations are given in table 1.

The following conclusions may be drawn from these data: (1) The cell and gum production of the crown-gall organism was slightly higher than that of the hairy-root organism. (2) The major part of the metabolic products remained in the culture medium after removal of the carbon dioxide, cells, and alcohol-

precipitable gum. Seventy to 80 per cent of the carbon in the fermented glucose formed unknown products. (3) Less glucose was recovered in known products from the cultures of the crown-gall organism than from those of the hairy-root cultures.

A search for products which, on the basis of the above, must be present resulted in the isolation of two unidentified products from cultures of each organism. An outline of the procedures employed and some of the characteristics of these products follow.

TABLE 1

*Glucose fermentation by the crown-gall and hairy-root organisms, and carbon distribution of the products*

ANALYTICAL DETERMINATIONS	CROWN-GALL ORGANISM				HAIRY-ROOT ORGANISM			
	Experiment 13		Experiment 14		Experiment 5		Experiment 6	
	grams per 100 cc.	per cent	grams per 100 cc.	per cent	grams per 100 cc.	per cent	grams per 100 cc.	per cent
Glucose fermented* . . . . .	0 847	42 30	0 806	40 30	0 657	32 80	0 700	35 0
Carbon in glucose fermented . . . . .	0 340		0 322		0 262		0 280	
Carbon in CO <sub>2</sub> . . . . .	0 0053	1 60	0 0053	1 70	0 0597	22 70	0 0606	21 7
Carbon in cells and gum . . . . .	0 0390	11 40	0 060	18 60	0 025	9 50	0 03	10 7
Carbon in products determined . . . . .	0 0443	13 00	0 0653	19 30	0 0847	32 20	0 0906	32 4
Carbon not accounted for . . . . .	0 295	87 00	0 257	80 70	0 177	67 80	0 189	67 6
Carbon in culture at beginning . . . . .	0 836		0 836		0 826		0 826	
Carbon in culture at end plus carbon in CO <sub>2</sub> . . . . .	0 856		0 830		0 803		0 816	

\* The medium contained 2.0 grams glucose per 100 cc. before fermentation.

#### ISOLATION OF GUM AND GUM-LIKE PRODUCTS

Twenty to 40 liters of the basal medium containing 3 per cent glucose were incubated at room temperature for thirty days and then worked up for gum and gum-like products.

*Gum.*<sup>2</sup> Precipitation of the gum was effected with alcohol or

<sup>2</sup> A different medium in large quantities was used for the preparation of some of the samples of gum. This consisted of magnesium sulfate (MgSO<sub>4</sub>·7H<sub>2</sub>O), 0.2 gram; sodium chloride (NaCl), 0.2 gram; calcium chloride (CaCl<sub>2</sub>), 0.1 gram; potassium phosphate (K<sub>2</sub>HPO<sub>4</sub>), 0.2 gram; potassium nitrate (KNO<sub>3</sub>), 5.0 grams; and glycerol, 10.0 grams.

The bacteria were removed by filtration from several hundred liters and the filtrate was concentrated to about one-tenth its volume. This work was done by Mulford Biological Laboratories, Sharp and Dohme, to whom grateful acknowledgment is made.

acetone, and with barium hydroxide. The hydroxide treatment is preferable when it is desired to isolate other metabolic products from the filtrate, whereas the first procedure is more convenient for the isolation of the gum.

The gum was purified by dissolving it in water and reprecipitating with alcohol acidified with hydrochloric acid. The excess alcohol was squeezed out and the gum dried over calcium chloride in a vacuum desiccator. The product was white, difficultly soluble in water, non-reducing to Fehling's solution, and precipitable from aqueous solutions by barium hydroxide and basic lead acetate. Such preparations were free from nitrogen but contained from 5 to 30 per cent ash. A preparation containing only 0.36 per cent ash was obtained by electrodialysis.

When the gum was hydrolyzed with 2 per cent HCl or 5 per cent  $H_2SO_4$ , reducing values calculated as anhydroglucose accounted for 72 to 98 per cent of the ash-free material. Uronic acid and total pentosan determinations gave figures of about 5 per cent for both components. Qualitative tests on the gum-hydrolysate for mannose, fructose and galactose were negative. Evidence that the reducing substance consisted mainly of glucose was obtained by the isolation and characterization of this sugar.

Ten grams of crown-gall gum, containing 0.36 per cent ash, were hydrolyzed with 5 per cent  $H_2SO_4$  at 15 pounds pressure for 5 hours. The solution was neutralized with hydroxide-free barium carbonate, heated for 20 minutes at 60°C., and the barium sulphate filtered off and washed with hot water. The filtrate was concentrated under reduced pressure at 60°C. to a thick syrup which was extracted with a number of portions of hot 95 per cent alcohol or hot absolute methyl alcohol. The alcoholic extract was decolorized with norit, concentrated to a thick syrup and two volumes of glacial acetic acid were added. After one week in the refrigerator the crystals which had formed were filtered off, washed with glacial acetic acid and recrystallized from a small amount of water and acetic acid. The yield was about 4 grams. Further concentration of the filtrate yielded about 2 grams more of crystals. The specific rotation of a dried sample of the first crop was  $[\alpha]_D^{25} = +52.0^\circ$  (C. = 65.8 mgm./cc.). The specific

rotation of glucose is  $+52.5^\circ$ . The osazone of the sugar and that of glucose prepared at the same time melted at  $204^\circ\text{C}$ . (uncorrected).

From these data it appears that the gum consists mainly of anhydro-glucose units. Gum prepared from the glycerol-salts medium had approximately the same composition and yielded the same products on hydrolysis as that from the glucose-salts medium.

*Gum-like products.* Since such a large portion of the fermented glucose remained unaccounted for, a search for metabolic products, other than carbon dioxide, cells and gum, was made. Substances, as yet incompletely characterized, but which resemble the bacterial gum in certain respects were found.

The cells and pellicle were filtered from 20 to 30 liters of culture and the bacterial gum was precipitated by addition of a saturated solution of barium hydroxide. An appreciable excess of barium hydroxide solution (about 1 to 2 liters for every 30 liters of culture medium) was added, and the mixture was allowed to stand for thirty minutes. The bulky precipitate was removed by filtration, washed with water, and the washings added to the filtrate. Besides the barium salts of the gum the precipitate contained phosphate, sulfate and carbonate.

The filtrate was treated with carbon dioxide until it was nearly acid to phenolphthalein. The precipitated barium carbonate was filtered off, the filtrate acidified with acetic acid and evaporated under reduced pressure to a thick syrup. An excess of acetic acid was avoided, since it exerted a definite solvent action on the lead precipitate obtained later.

A saturated solution of basic lead acetate was added, with agitation, to the thick syrup. A gelatinous precipitate was formed, which on standing in contact with excess basic lead acetate became flocculent. The precipitate was filtered off and washed with water. In order to remove the glucose it had adsorbed, the precipitate was repeatedly suspended in 60 per cent alcohol and settled by centrifuging. Great difficulty was experienced in removing the glucose, and the washing process, though tedious, was more satisfactory than repeated precipitation because the

latter caused serious loss of material. In later experiments the washing process was shortened by placing the lead salt in collodion bags and dialyzing in running water for two weeks.

The washed salt was dissolved in dilute acetic acid and the lead precipitated with hydrogen sulfide. The filtrate and washings were combined and evaporated under reduced pressure until all but a small amount of the acetic acid had been removed. The clear glassy material was dissolved in a minimal quantity of water and the solution saturated with cupric acetate. Ninety-five per cent alcohol was added, which precipitated the copper salt of the product. In the presence of too large an excess of acetic acid the copper salt was precipitated in the form of a heavy syrup. The tendency to precipitate as a syrup was especially marked with material from the crown-gall organism.

The precipitate was allowed to settle and the supernatant alcohol decanted. The remainder of the material was filtered and the precipitate washed until the washings gave no test for glucose.

The filtrate from the lead precipitation was delead with hydrogen sulfide and the filtrate and washings were evaporated under reduced pressure to a thick syrup to remove as much acetic acid as possible. The syrup was diluted until it could be handled conveniently and saturated with copper acetate. On adding alcohol a precipitate formed. This was filtered and washed as described above.

The above method of isolation is summarized in figure 7.

The metabolic products nos. 2 and 3 have not been completely characterized. They were obtained in the free state in the form of a hard glassy mass by evaporating their aqueous solutions under reduced pressure, or in the form of a sticky gum when concentrated solutions were treated with alcohol. On standing in contact with alcohol the precipitate would slowly redissolve. Oxidation with nitric acid yielded oxalic acid. No reducing sugars were formed on hydrolysis with either hydrochloric or sulfuric acids.

Since the substance precipitated by basic lead acetate from cultures of the hairy-root organism (metabolite no. 2) could be isolated and purified with less effort than the corresponding

product from the crown-gall organism, it was selected as the most suitable for experiments designed to secure information concerning the nature of the material. Acetylation, benzylation, and methylation yielded syrups that could not be crystallized. The 3-5 dinitrobenzoate, prepared by heating the metabolic product with 3-5 dinitrobenzoyl chloride, was likewise a syrup and could not be crystallized. These syrups differed from the original material in respect to solubility. The original material was

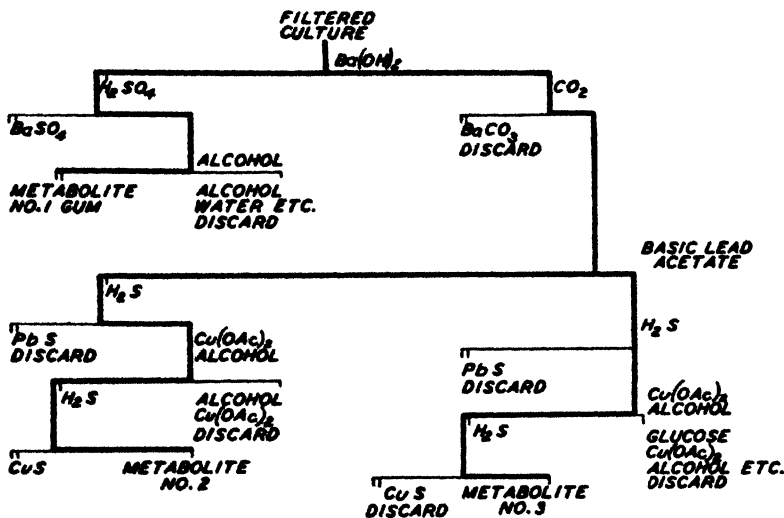


FIG. 7. DIAGRAMMATIC REPRESENTATION OF THE PROCEDURE USED TO ISOLATE CERTAIN METABOLIC PRODUCTS OF THE HAIRY-ROOT AND CROWN-GALL ORGANISMS

soluble in water and only difficultly soluble in alcohol, while the acetyl and benzoyl derivatives were insoluble in water and soluble in alcohol and other organic solvents.

Since acid phthalates are crystalline in many cases, an attempt was made to prepare the acid phthalate of the substance by heating it with phthalic anhydride to 200°C. Nothing could be isolated from the mixture except phthalic anhydride.

Reduction with hydroiodic acid and phosphorous yielded only traces of a volatile oil, while the remainder of the material refused to crystallize and could not be distilled.



The acetone compound of the metabolite was prepared by treating it with purified acetone, containing hydrogen chloride as a catalyst, for eight days at 36°C. At the end of this time the acetone was removed and the residue dissolved in alcohol. The alcoholic solution was boiled with decolorizing charcoal and filtered. Nothing could be crystallized from the solution. Oxidation of the acetone compound with alkaline permanganate yielded no definite product.

#### DISCUSSION

While distinct differences exist in their nitrogen metabolism (Sagen, *et al.* 1934), the close relationship of the crown-gall and hairy-root organisms, as indicated by their similar pathogenicity, is reflected in their carbon metabolism. With the exception of the production of acid by the hairy-root organism, only quantitative differences have been found in their behavior during these studies. The organisms are quite similar in their abilities to ferment glucose. The hairy-root organism is, however, able to ferment high concentrations of glucose more rapidly than the crown-gall organism.

In all cases the hairy-root organism produced more carbon dioxide than the crown-gall organism. On agar media the hairy-root organism produced carbon-dioxide rather rapidly for the first seven days, then less rapidly for the remainder of the period. The crown-gall organism produced carbon dioxide at a fairly steady rate during the entire course of the fermentation.

The production of acid from glucose by the hairy-root organism is also a useful method of distinguishing it from the crown-gall organism. It is of interest to note that pyruvic acid, which is produced by hairy-root bacteria has been reported (Hitchcock, 1935) to induce a growth response in plants. However, the evidence is inadequate to determine whether this acid is involved in the growth response induced in the host by the hairy-root organism.

While no proof exists that the various metabolites discussed are produced in the host plant, it is not improbable that they do occur in plant tissue. It remains for further study to determine

whether these substances play a rôle in the peculiar type of cell division found in the tissue surrounding the bacterial pockets.

#### SUMMARY

Quantitative data have been obtained regarding the fermentation of glucose, the rates of fermentation, the amounts of carbon dioxide produced, and the carbon distribution of the metabolic products of the crown-gall and hairy-root organisms.

The amount of glucose fermented in a liquid medium by the hairy-root and crown-gall organisms may be raised by increasing the concentration of yeast infusion or by the addition of one per cent phosphates. Agar media were more satisfactory for fermentation than liquid media.

The hairy-root organism was able to ferment higher concentrations of glucose more rapidly than the crown-gall organism.

The hairy-root organism produces approximately ten-fold as much carbon dioxide as the crown-gall organism.

Acetic and pyruvic acids were identified as metabolic products of the hairy-root organism.

From 13 to 20 per cent of the carbon of the fermented glucose was recovered as carbon dioxide, cells, and gum from cultures of the crown-gall organism and approximately 30 per cent was recovered from cultures of the hairy-root organism. Seventy to 80 per cent of the sugar fermented goes to form other products, some of which have been isolated and partly characterized. These metabolites differ from the bacterial gum in that they do not yield reducing sugars on hydrolysis.

The bacterial gum consisted chiefly of glucose units (72 to 98 per cent) and small amounts (5 per cent) of uronic acid. Qualitative tests for other hexoses were negative.

The writers are indebted to Dr. Rudolph Nagy for his collaboration in the preparation and analysis of the gum produced by the organisms.

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# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## EASTERN PENNSYLVANIA CHAPTER

A SYMPOSIUM ON HEMOLYTIC STREPTOCOCCI, PHILADELPHIA COUNTY MEDICAL  
SOCIETY BUILDING, PHILADELPHIA, PENNSYLVANIA, MARCH 23, 1937

**THE COLONY FORM OF HEMOLYTIC STREPTOCOCCI FROM CLINICAL SOURCES.** *David Lackman*, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia.

The colony forms of the strains of hemolytic streptococci received in our laboratory from recent clinical cases are summarized in table I. The media used was the moist neopeptone buffered horse-blood agar recommended by M. H. Dawson. The colonies were examined with a colony microscope after 18-20 hours incubation at 37°C. No significant correlation of colony form with mortality was found as is shown in table II.

**SURFACE COMPOSITION OF MUCOID, GLOSSY AND ROUGH VARIANTS OF HEMOLYTIC STREPTOCOCCI.** *Stuart Mudd and Horace Pettit*, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia.

The cell surfaces of mouse-virulent hemolytic streptococci of Lancefield Group A, grown for 18 hours in buffered neopeptone infusion broth, consist predominantly of the reversibly oxidizable labile antigen. (Streptococcus labile antigen has been described in summary: *Proc. Am. Phil. Soc.*, 1937, 77, 463-6; detailed publications are in

preparation.) Agglutination and phagocytosis of, and mouse-protection against, these virulent forms occur primarily in response to type-specific antibody against the labile antigen.

In the cell surfaces of variants of glossy colony form the quantity of labile antigen present is much reduced, and a correspondingly larger part of the surface is composed of the group-specific carbohydrate C. Cross-absorption experiments between labile antigens of mucoid and glossy variants have detected quantitative, but not qualitative differences in their capacity to absorb antibodies against labile antigen. Glossy variants may be caused to agglutinate and undergo phagocytosis in response either to type-specific antibody against labile antigen or group-specific antibody against the carbohydrate C. In a rough variant, labile antigen has not been detected with certainty, the surface being predominantly composed of C.

There is evidence to suggest the occurrence also of the stable hemolysin-leucocidin in the surfaces especially of the glossy and rough variants, but this evidence needs corroboration.

**ISOLATION AND SPECIFICITY OF THE LABILE ANTIGEN OF STREPTOCOCCUS HEMOLYTICUS (GROUP A).** *E. J. Czarnetzky, Stuart Mudd, H. Pettit*

and David Lackman, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia.

A labile antigen of Group A hemolytic streptococci has been isolated in pure form by procedures already described. (Proc. Am. Phil. Soc., 1937, 77, 463-6.) Analysis by Dr. R. W. Wyckoff in the ultracentrifuge has

shown on injection gives rise only to antibodies against labile antigen, but labile antigen can absorb from antisera prepared with whole organisms antibodies against the homologous type specific hapten M and the group specific hapten C. Labile antigen on hydrolysis yields the haptens M and C, but the haptens even when mixed together can not absorb antibody against labile antigen.

It may be concluded that streptococcus labile antigen is a compound of M and C which is so oriented in the cell surface that the type-specific portion of the molecule is reactive and the group-specific portion partially or wholly masked.

**A STABLE HEMOLYSIN-LEUCOCIDIN FROM  $\beta$ -HEMOLYTIC STREPTOCOCCI.**  
*E. J. Czarnetsky and Isabel Morgan*, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia.

A hemolysin, active to a dilution of 1 in 40,000, can be isolated from  $\beta$ -hemolytic streptococci by extraction of the lyophile dried organisms with moist ethyl ether. It is stable to heat, oxygen and changes in hydrogen ion concentration within the physiological range. It has been isolated in a chemically pure state, and has a molecular weight of 2260. A derivative with a molecular weight of 720 can be crystallized as the sodium salt from water or alcohol. The derivative consists of three molecules of sugar or sugar acids and at least one phenolic hydroxy group. The native form consists of 3 molecules of the crystallisable derivative plus one molecule of phosphorus and three molecules of nitrogen.

The presence of the native form or the crystallizable derivative inhibits polymorphonuclear leucocytes from reducing methylene blue. The two forms

TABLE I

CLINICAL DIAGNOSIS	NUMBER OF CASES	COLONY FORM OF STRAINS ISOLATED		
		Percentage		
		Mucoid	Matt	Glossy
Blood stream septicemia..	18	50	27	23
Mastoid.....	8	63	25	12
Acute sore throat.....	20	40	40	20
Scarlet fever.....	25	8	40	52
Local lesions.....	23	22	48	30
Puerperal fever	16	50	31	19
Erysipelas				
Meningitis				
Pneumonia				

TABLE II

COLONY FORM	NUMBER OF CASES	PERCENTAGE MORTALITY
Mucoid.....	43	21
Matt.....	41	17
Glossy.....	23	26

shown the antigen of each of two types to consist of a single molecular species.

The labile antigen is group specific (i.e., not specific for type) in the precipitin test; on the other hand agglutination and phagocytosis of strains whose surface is predominantly composed of labile antigen, either by antiserum prepared against whole organisms or against labile antigen alone, is relatively type-specific. Labile anti-

are active to approximately the same degree. The native form acts as a hapten, giving precipitates with sera against any  $\beta$ -hemolytic streptococci, including those of groups other than Lancefield Group A. The crystallizable form is inactive serologically. Neither form has produced antibodies when injected by itself into rabbits.

**CONVALESCENT SCARLATINAL SERUM IN THE PREVENTION AND TREATMENT OF STREPTOCOCCAL DISEASES.** *Aims C. McGuinness and Joseph Stokes, Jr.*, Department of Pediatrics, University of Pennsylvania, and the Childrens Hospital, Philadelphia.

Pooled lyophile convalescent scarlet fever serum has been employed in the prevention of scarlet fever in 545 exposed individuals. Scarlet fever developed in only 8 of these cases. In 6 of the 8 cases the disease developed between the tenth and twentieth day following the injection of serum, and in all cases the exposure was intimate and continuous. In view of the above, and of the fact that reversal of the Dick tests following serum injection lasts for only 10 or 12 days, it would seem advisable to repeat the serum injections every 10 days during intimate continuous exposures.

Lyophile convalescent scarlet fever serum has been used in the treatment of 95 cases of scarlet fever. In 15 of these cases the disease was so mild that the value of the serum therapy could not be determined except to say that none of the mild cases developed any complications. Of the remaining 80 cases, 68 responded favorably to the serum with a rapid fall in temperature, decrease in toxemia, and a fading rash. The majority of the cases treated received a single injection of from 30 to 60 cc. of serum. In several instances the initial dose was repeated once and

in one case twice. Complications developing in treated cases were few and of mild degree.

Lyophile convalescent scarlet fever serum has been employed in the treatment of a small series of cases of hemolytic streptococcal infection other than scarlet fever. Among the conditions treated were acute pharyngitis, acute laryngitis, cervical adenitis, mastoiditis, puerperal sepsis, and meningitis. While no definite conclusions may be drawn, the response to the serum appeared to be very definite in a number of these cases, especially in the pharyngitis-laryngitis group. One case of meningitis of hemolytic streptococcus origin made an uneventful recovery following the combined intramuscular and intrathecal injection of this type of serum.

**CHEMOTHERAPY IN STREPTOCOCCAL INFECTIONS.** *D. Sergeant Pepper, E. J. Czarnetzky and I. S. Ravidin*, Departments of Medicine, Bacteriology and Surgery, University of Pennsylvania, Philadelphia.

A simple organic compound, sulfanilamide, has been demonstrated to be efficacious in preventing the death of mice infected with  $\beta$ -hemolytic streptococci. This compound and various derivatives of about equivalent activity have been used in the treatment of approximately 175 cases of streptococcal diseases in various hospitals in the Philadelphia district. The cases treated include those of septicemia, puerperal sepsis, peritonitis, rheumatoid arthritis, erysipelas, sore throat, cellulitis, empyema, mastoiditis, osteomyelitis, and miscellaneous local infections.

Even with the limited number of cases treated in each group, and the lack of controls, the results have been sufficiently impressive so that it is felt

that administration of sulfanilamide is indicated in all cases of hemolytic streptococcal infection. The drug is not toxic when administered properly,

but can be decidedly toxic when given in excessive amounts or to individuals who are, or become, abnormally sensitive to the drug.

### CONNECTICUT VALLEY BRANCH

SMITH COLLEGE, NORTHAMPTON, MASSACHUSETTS, MAY 15, 1937

AN UNDESCRIBED SEROTYPE OF *SALMONELLA* ISOLATED FROM CHICKS. *Erwin Jungherr and Carl F. Clancy*, Storrs Agricultural Experiment Station, Storrs, Connecticut.

Four strains of a *Salmonella* organism not conforming to any type in the Kauffmann-White scheme were isolated from a specimen lot of 15 chicks affected with omphalitis. The somatic structure was shown to be VI, VII (group C). In H agglutination tests the organism appeared monophasic and showed partial reactions with antisera of *Salmonella oranienburg* (m t), *S. senftenberg* (g s), *S. enteritidis* (g o m), and *S. derby* (f g). Mirror absorption tests indicated the presence of factors g o m s t of which g o s possessed agglutinating but not complete absorbing power. A phase variation was noted in that colonies consisting of rods showed, primarily, factors g m t, whereas colonies showing filamentous elements possessed all the specific factors. Dr. P. R. Edwards of Kentucky, to whom a strain was forwarded, likewise recognized the H relationship to *S. senftenberg*, *S. oranienburg*, and *S. enteritidis* and kindly called our attention to the description of *S. montevideo* (Hormaeche, *Ztschr. Hyg.*, 1937, 119: 453), isolated from cases of infant diarrhea. He observed complete absorption of a chick-strain serum by *S. montevideo*. This result was confirmed in our laboratory. Accordingly, the present antigenic conception of the chick organism which resembles *S.*

*montevideo* is VI, VII, g o m s t, -, with factors g o s deficient in absorbing capacity.

THE PRESENCE OF THE Vi ANTIGEN IN EXTRACTS OF *EBERTHELLA TYPHOSA*. *Elinor Van Dorn Smith*, Department of Hygiene and Public Health, Smith College, Northampton, Mass.

Toxic filtrates of *Eberthella typhosa* were prepared by heating washed, aqueous suspensions of 18-hour beef heart infusion agar cultures for four hours at 60 C., centrifuging, and filtering the supernatants through Berkefeld "N" candles. Five-tenths of a milliliter was the average lethal dose of the different extracts for mice, while 0.03 to 0.08 ml. per kilogram weight was frequently lethal for rabbits. Repeated injections of small doses developed a protection in rabbits against subsequent injections of at least 30 minimum lethal doses.

These extracts, which possessed unusual toxicity and immunizing potency, were found to come from strains containing the Vi antigen. Although this factor could not be shown in the filtrates by the precipitation test, Vi agglutinins were developed in rabbits injected with raw, boiled, or autoclaved extracts, thus demonstrating the thermostability of the agglutinogenic activity of the Vi fraction.

A subsequent comparison of extracts from Vi positive and negative cultures revealed no significant differences in toxicity. The presence or absence of

antibodies for this fraction in extract-injected rabbits did not affect the neutralizing powers of their serums in vitro or their immunity to the endotoxins. The Vi fraction, although present in extracts of *E. typhosa*, apparently played no significant rôle in their toxicity.

**HUMAN SCARLATINAL STREPTOCOCCI IN MONKEYS.** *Paul L. Boisvert, M.D.*, Department of Pediatrics, Yale University, School of Medicine, New Haven, Connecticut.

Hemolytic streptococci were recovered from four *Rhesus* monkeys. Three had spontaneous infections (two: facial erysipelas; one: an axillary abscess) and the fourth developed a purulent peritonitis following the intraperitoneal injection of Dial. The organisms were seen in the pus from the peritoneum and abscess, and in all cases a culture of the lesion yielded a pure growth of hemolytic streptococci, which were bile-insoluble and produced a soluble hemolysin. By precipitin tests, all strains fell into Lancefield's group A, and their biochemical and cultural characteristics were those of human pathogenic organisms. They fermented lactose, salicin, and trehalose, but not mannitol or sorbitol. They did not hydrolyse sodium hippurate or reduce methylene blue milk. Growth on 10% bile blood agar was very poor or absent, and there was no growth on 40% bile blood agar. All lysed a human plasmatoc rapidly and produced a human erythrogenic toxin. The skin test dose with two of the strains was 0.1 c.c. of a 1:10,000 dilution of filtrate, and 0.1 c.c. of a 1:100 dilution with the other two strains. The four toxins were neutralizable with scarlatinal antitoxin. By the available tests these four strains were found to be indistinguishable

from those found in scarlet fever and other severe human streptococcal diseases.

**BACTERIOLOGICAL STUDY OF MACHINISTS' CUTTING COMPOUND.** *James E. Fuller*, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.

Samples of cutting compound from an industrial plant were examined on three occasions, at intervals of two weeks. The predominating organisms were members of the coli-aerogenes group. All fermented lactose with the production of acid and gas in 24 hours. In their growth on Endo's medium they resembled *Aerobacter aerogenes* rather than *Escherichia coli*. Their reactions to the Voges-Proskauer, methyl red, sodium citrate and uric tests, and indol production were determined; all strains were then classified as intermediates of the group. The cutting compound consisted of an abrasive in light oil, and water was employed to give it volume. An examination of the water supply employed indicated that the supply was the source of these organisms. No staphylococci were recovered on either blood agar or nutrient agar plates, which indicated that the numbers of these organisms were relatively small, if they were present at all. Some organisms of the *Bacillus subtilis* group were observed.

**DEMONSTRATION OF A MULTIPLE PIPETTING MACHINE.** *W. N. Platridge and L. F. Williams*, Department of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Connecticut.

A machine capable of placing measured amounts of agglutination antigen in test tubes at the rate of about 130 tubes per minute was demonstrated.





# FIBRINOLYTIC, ANTICOAGULATING AND PLASMA-CLOTTING PROPERTIES OF STAPHYLOCOCCI

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In 1908, Much showed that plasma which had been coagulated by the plasma-clotting factor of staphylococci, may become redissolved. He attributed the lysis of the plasma clot to the action of a staphylococcus fibrinolysin. His observations were confirmed by Kleinschmidt (1909), Gonzenbach and Uemura (1916), Gratia (1921), Aoi (1932), Gengou (1933), Vanbreuseghem (1934), Madison (1935), and Fisher (1936). The production of powerful fibrinolysin by hemolytic streptococci was demonstrated by Tillett and Garner (1933). Confirming Tillett and Garner's results, Dennis and Berberian (1934) found a second factor produced by hemolytic streptococci which inhibits coagulation of plasma. Both factors were considered by these authors to be antigenic. Furthermore, they reported that one strain of *Streptococcus viridans* produced the anticoagulant. According to Tunncliffe (1936), there is a relation between the production of anticoagulant by *Streptococcus viridans* and its smooth phase. Neter and Witebsky (1936) showed that the production of bacterial anticoagulants is not limited to *Streptococcus hemolyticus* only. They found that *Streptococcus viridans*, enterococci, pneumococci of various types, some strains of *Escherichia coli*, *Pseudomonas pyocyaneas* and others may produce an anticoagulant. In a subsequent paper (Witebsky and Neter, 1936) the differences in the properties of the fibrinolysin and the bacterial anticoagulants were described. The authors came to the conclusion that both factors are entirely independent of each other. Recently, Dart (1936) reported that the streptococcus anticoagulant may be

separated from the streptococcus fibrinolysin by means of alcohol extraction. The streptococcus fibrinolysin and the bacterial anticoagulants are not artificial products obtained in vitro only, since both factors were found to be present in human exudates (Neter and Witebsky, 1936; Neter, 1936; Neter and Young, 1937).

The following experiments are concerned with a comparative study of the production of fibrinolysin and anticoagulant by staphylococci in vitro and in vivo, and with the relation of the staphylococcus anticoagulant to the plasma-clotting factor of these microorganisms.

#### TECHNIC

Various strains of staphylococci were isolated from human lesions; they were tested for pigment production on plain agar plates and for hemolytic activity on 5 per cent human-blood agar plates. For the determination of gelatin liquefaction, nutrient gelatin (Difco) was employed.

For the production of fibrinolysin and anticoagulant, the respective strains of staphylococci were cultured in plain and 1-per-cent-glucose infusion broth for 18 hours at 37°C.<sup>1</sup> The cultures were centrifuged and the supernatant fluids tested according to the technic of Tillett and Garner (1933). The plasma used for the experiments was prepared by mixing 10 cc. of blood (human, rabbit or guinea pig blood) with 1 cc. of a 2 per cent potassium oxalate solution. The blood was shaken thoroughly and centrifuged. For the demonstration of either the fibrinolysin or the anticoagulant, the supernatant fluid of the respective culture in serial dilutions (volume 0.5 cc.) was mixed with 1 cc. of 1:5 dilution of plasma; then 0.25 cc. of a 0.25 per cent calcium chloride solution in normal saline was added; the tubes were shaken thor-

<sup>1</sup> The respective broths were always tested and found to be lacking in coagulating and anticoagulating properties in the order of the experiment described. For comparative studies, culture media prepared by the Digestive Ferments Company were also employed. Brain heart infusion, heart infusion broth and veal infusion medium gave practically the same results as the meat infusion broth prepared in this laboratory. Extract broth, however, sometimes inhibited the clotting of human plasma and, therefore, could not be used for the study of the anticlotting factor of bacteria.

oughly and kept at 37°C. The results were read at various intervals. The fibrinolysin is characterized by dissolution of the plasma clot, the anticoagulant by its continuous inhibition of the coagulation of the plasma.

For the demonstration of the plasma-clotting factor of staphylococci, serial dilutions of the supernatant fluid of the respective cultures in the volume of 0.5 cc. were mixed with 0.25 cc. of plasma, incubated at 37°C. and read at various intervals.

### RESULTS

Sixty strains of staphylococci of human origin were examined. Of 43 strains of *Staphylococcus aureus-hemolyticus*, 2 produced the fibrinolysin in plain infusion broth, 35 the anticoagulant in 1-per-cent-glucose infusion broth; 4 strains produced the fibrinolysin as well as the anticoagulant and 2 lacked both properties. Seven strains of *Staphylococcus albus-hemolyticus* were tested; 1 was found to produce the fibrinolysin, 4 the anticoagulant and 2 strains failed to show fibrinolytic or anticlotting properties. Of 10 strains of non-hemolytic staphylococci (*S. aureus* and *S. albus*), none produced the fibrinolysin, 5 showed anticlotting properties and 5 were negative. While the majority of staphylococci isolated from human lesions showed anticlotting properties, only a relatively small percentage produced the fibrinolysin, in contradistinction to hemolytic streptococci. According to Aoi (1932) and Madison (1935), however, a higher percentage of fibrinolytic staphylococci is obtained when the isolated fibrin technic is employed; or, as in the experiments of Fisher (1936), when the observation period is extended over several days. In this connection it may be mentioned that on some occasions, staphylococcus fibrinolysin was found to be effective toward plasma coagulated by means of the plasma-clotting factor of staphylococci, but failed to dissolve the same plasma when clotted by the addition of calcium chloride solution.

Experiments were carried out to determine whether or not a relationship exists between the production of fibrinolysin and anticoagulant by staphylococci on the one hand, and their ability to liquefy gelatin on the other. The majority of strains of

staphylococci isolated from human lesions liquefied gelatin and produced the staphylococcus anticoagulant. A few strains, however, did not liquefy gelatin within 3 days, but produced the anticoagulant. Two fibrinolytic strains of staphylococci tested, were found to liquefy gelatin.

The properties of *Staphylococcus fibrinolysin* of human strains parallel those of the *Streptococcus hemolyticus* fibrinolysin with the exception that the fibrinolysin obtained from staphylococci acts more slowly and may be effective toward human as well as animal plasma. This latter observation corresponds to the findings of Madison and Dart (1936), who reported that some veterinary staphylo-fibrinolysins were effective also toward human plasma. The antigenicity of the staphylococcus fibrinolysin could be demonstrated in the following ways: (1) Staphylococcus antiserum (Staphylococcus Antitoxin Lederle) neutralized specifically the staphylococcus fibrinolysin as did the serum of a 12-year-old boy with osteomyelitis of the right thigh of one year duration; (2) the plasma of this patient was found to be resistant toward the staphylococcus fibrinolysin in contradistinction to plasma obtained from normal individuals and from two children with scarlet fever. The plasma of the above patient, suffering from osteomyelitis, however, was susceptible toward the *Streptococcus hemolyticus* fibrinolysin.

The *staphylococcus anticoagulant* has the same properties as the anticoagulant produced by other microorganisms: it inhibits coagulation continuously; it is effective toward human as well as animal plasma; it is produced in broth containing carbohydrates, but not in plain infusion broth. When staphylococci were cultured in broths containing various carbohydrates, such as glucose, lactose, maltose, sucrose and mannitol, different strains produced the anticoagulant in different carbohydrate media. The effectiveness of the staphylococcus anticoagulant may be equally inhibited by either normal serum or spinal fluid, proving that a factor other than an antibody-function is responsible for this inhibition.

Experiments were carried out to elucidate whether or not the staphylococcus anticoagulant is antigenic. For this purpose,

*Staphylococcus Antitoxin* (Lederle) and the serum of the above-mentioned patient with chronic osteomyelitis were tested; both sera failed to inhibit the effectiveness of the staphylococcus anticoagulant to a greater extent than did normal serum or spinal fluid. Moreover, coagulation of the plasma of the above patient with osteomyelitis was inhibited in the presence of the staphylococcus anticoagulant. It may be concluded, therefore, that the staphylococcus anticoagulant is lacking in antigenicity.

According to their action on plasma, staphylococci may be classified into one of the following four groups: (1) Staphylococci may produce the specific fibrinolysin; (2) they may form an anti-clotting principle; (3) they may possess both properties or (4) they may lack both properties. The simultaneous presence of staphylococcus anticoagulant and fibrinolysin in glucose broth could be demonstrated in two ways: (1) When the supernatant fluid of the glucose broth is titrated, the undiluted broth inhibits coagulation, while the broth in higher dilutions causes lysis of the plasma clot. (2) When undiluted glucose broth is mixed with plasma which has been diluted with normal spinal fluid instead of saline solution, fibrinolysis occurs and not inhibition of plasma coagulation, because normal spinal fluid inhibits the effectiveness of the anticoagulant only. The question arises whether a relation exists between the production of fibrinolysin or anticoagulant and the pathogenicity of staphylococci for man. The respective experiments may be summarized as follows: Staphylococci producing the fibrinolysin as well as those producing the anticlotting factor were found as causative agents in abscesses, exudates and in the blood stream in cases of staphylococcus sepsis. Both types of staphylococci, therefore, may be virulent for man and may invade the blood stream.

In order to determine whether the staphylococcus anticoagulant and the staphylococcus fibrinolysin may be found in vivo, purulent exudates were examined. The staphylococcus fibrinolysin could be demonstrated in the supernatant fluid of a staphylococcus empyema in two cases as well as in the purulent exudate of a case of osteomyelitis. The strains obtained from these lesions also produced the staphylococcus fibrinolysin in vitro. The

staphylococcus anticoagulant too may be present in exudates caused by staphylococci; it was found in the supernatant fluid of a staphylococcus empyema of a patient who suffered from staphylococcus sepsis; the strains isolated from the blood stream as well as from the empyema fluid in pure culture produced the staphylococcus anticoagulant also in vitro.

Staphylococci are capable of clotting human as well as animal plasma. This plasma-clotting property has been known for many years and was extensively studied by Loeb, Much, Kleinschmidt, Gross, Gratia, Chapman, Fisher and others. A review of this subject is given in the article by Fisher (1936). The question, whether the plasma-clotting factor of staphylococci might be of significance in the development and course of staphylococcus infections was recently discussed by Chapman, Berens, Peters and Curcio (1934), by Menkin and Walston (1935) and by Pijoan (1935).<sup>\*</sup> It is of interest, therefore, to determine whether or not the staphylococcus coagulant may be produced in natural staphylococcus infections. For this purpose, exudates obtained from human beings suffering from staphylococcus infections were examined; they were centrifuged immediately after the specimens were obtained. The supernatant fluid in decreasing amounts (volume 0.5 cc.) was mixed with 0.25 cc. of human as well as guinea pig and rabbit plasma. Several specimens even in dilutions up to 1:100 caused coagulation of human and animal plasma. In one case of staphylococcus pericarditis, the supernatant fluid of the exudate (dilution 1:2) caused clotting of human and guinea pig plasma within 10 minutes. In this connection, it may be mentioned that the exudates in two cases also contained the fibrinolysin and, in one case, the anticoagulant besides the plasma-clotting factor of staphylococci. It follows from these experiments that the staphylococcus coagulant may be found in vivo and may be present simultaneously with the staphylococcus anticoagulant or fibrinolysin.

In view of the fact that staphylococci may cause as well as inhibit coagulation of plasma, the quantitative relation between staphylococcus anticoagulant and the plasma-clotting factor of these microorganisms was examined. The following protocol

represents a typical example: In part I of the experiment, the supernatant fluids of (a) plain broth, and (b) glucose broth cultures of *Staphylococcus aureus-hemolyticus* in decreasing amounts (volume 0.5 cc.) were mixed with 0.25 cc. of human plasma and incubated at 37°C. The resulting coagulation is recorded in

TABLE 1

*Relation of the plasma-clotting factor of Staphylococcus aureus-Hemolyticus to the staphylococcus anticoagulant*

Part I

DECREASING AMOUNTS OF SUPERNATANT FLUID OF BROTH CULTURE (VOLUME 0.5 cc.)	COAGULATION OF HUMAN PLASMA (0.25 cc.) BY SUPERNATANT FLUID OF CULTURE OF STAPHYLOCOCCUS GROWN IN	
	a Plain broth	b Glucose broth
(1) 0.5 cc.	Coagulation	No coagulation
(2) 0.05 cc.	Coagulation	Coagulation
(3) 0.005 cc.	Coagulation	Coagulation
(4) 0	No coagulation	No coagulation
(5) Broth control	No coagulation	No coagulation

Read after one hour incubation at 37°C.

Part II

DECREASING AMOUNTS OF SUPERNATANT FLUID OF BROTH CULTURE (VOLUME 0.5 cc.)	INHIBITION OF COAGULATION OF HUMAN PLASMA (1.0 cc. OF A 1:5 DILUTION) BY SUPERNATANT FLUID OF CULTURE OF STAPHYLOCOCCUS GROWN IN	
	a Plain broth	b Glucose broth
	After addition of 0.25 cc. CaCl <sub>2</sub>	
(1) 0.5 cc.	Coagulation	No coagulation
(2) 0.05 cc.	Coagulation	Coagulation
(3) 0.005 cc.	Coagulation	Coagulation
(4) 0	Coagulation	Coagulation
(5) Broth control	Coagulation	Coagulation

Read after one hour incubation at 37°C.

part I of table 1. In part II of the experiment, the same supernatant fluids were mixed with 1 cc. of 1:5 dilution of human plasma and 0.25 cc. of 0.25 per cent solution of calcium chloride. The mixtures were incubated at 37°C. and the resulting inhibition of coagulation is recorded in part II of table 1.



Table 1 shows that the plasma-clotting factor is present in the supernatant fluid of plain and glucose infusion broth cultures up to a dilution of 1:100. When undiluted glucose broth, however, is used, no coagulation is observed. Part II of the experiment offers an explanation of these findings: The anticoagulant, present in undiluted glucose broth counteracts the effectiveness of the plasma-clotting factor. The staphylococcus coagulant, however, is not destroyed by the anticoagulant since it can easily be demonstrated in higher dilutions of glucose broth. The antagonistic effect of the staphylococcus anticoagulant is not a specific one since the enterococcus anticoagulant was also found to inhibit the plasma-clotting factor of staphylococci.

#### DISCUSSION

Coagulation of exudates and dissolution of fibrin are important factors in bacterial infections, as for example the fibrinous exudate in pneumonia and the formation of fibrinous adhesions in certain cases of meningitis on the one hand, and the dissolution of fibrin in an infected thrombus on the other. A study of the influence of bacteria and their products on coagulation of plasma and dissolution of fibrin may, therefore, throw light on some phases in the development and course of bacterial infections.

Staphylococci from human sources may produce a fibrinolysin as well as a factor which inhibits coagulation of plasma. The staphylococcus fibrinolysin acts upon human as well as animal plasma clots. It is an antigen and can be neutralized by specific staphylococcus antisera. Patients with staphylococcus infections may develop a staphylococcus antifibrinolysin. The staphylococcus fibrinolysin resembles the streptococcus fibrinolysin which was first described by Tillett and Garner. It is, however, antigenically different.

The staphylococcus anticoagulant is produced by the majority of strains of staphylococci isolated from lesions of man. It has the same properties as the anticoagulant of other microorganisms. The anticoagulant inhibits the coagulation of plasma continuously; it is produced in culture media containing carbohydrates.

This anticoagulant differs distinctly from the staphylococcus fibrinolysin not only by its action upon plasma, but also by the fact that it is not antigenic.

The nature of bacterial anticoagulants is not known as yet. It is even questionable whether or not we are dealing with a uniform substance. Inhibition of coagulation of plasma may be achieved in different ways: On the one hand, calcium may be rendered ineffective; on the other hand, a substance may act upon fibrinogen or thrombin and its constituents. Further studies in this direction are under way. The fact that the bacterial anticoagulant is produced only in culture media which contain carbohydrates, suggests that the anticoagulating factor may be a derivative of carbohydrates. Various sugars are suitable for the production of the staphylococcus anticoagulant. Because of the importance of the carbohydrates for the development of the anticoagulant, the question arises whether or not the resulting acidity of the culture medium may be responsible for the inhibition of plasma coagulation. Preliminary experiments by the use of the quinhydrone-electrode, however, showed that a change of the pH cannot be the only factor. This is in agreement with the recent report of Dennis and Adham (1937).

Both bacterial fibrinolysin and anticoagulant are factors which may be of significance in natural infections. It was possible to demonstrate the staphylococcus fibrinolysin as well as the staphylococcus anticoagulant in exudates from human beings, proving that these substances may be produced in vivo. These results are in accord with the findings that fibrinolytic as well as anti-clotting staphylococci could be isolated as causative agents from lesions in man. In this connection, it is of interest to state that both types of staphylococci could also be isolated from the blood stream in cases of staphylococcus sepsis, proving that anticoagulating as well as fibrinolytic staphylococci may exhibit invasive properties.

Staphylococci may produce not only fibrinolysin and anticoagulant; they may also form a factor which causes clotting of human as well as animal plasma. This plasma-clotting factor of staphy-

lococci may be of importance for the development and course of staphylococcus infections. It was possible to demonstrate the staphylococcus coagulant in exudates obtained from human beings.

The possible interaction of these three factors: fibrinolysin, anticoagulant, and plasma-clotting factor, make an experimental analysis of staphylococcus infections difficult. The antagonistic effect of the plasma-clotting factor of staphylococci toward the staphylococcus anticoagulant could be demonstrated in vitro. The anticoagulant inhibits the plasma-clotting factor, but does not destroy it. The effectiveness of the plasma-clotting factor becomes manifest in dilutions in which the bacterial anticoagulant is no longer effective.

#### SUMMARY

1. Staphylococci from human sources may produce either fibrinolysin or anticoagulant, or they may simultaneously exhibit or lack both properties.

2. The staphylococcus fibrinolysin dissolves human as well as animal plasma clots. It can be specifically neutralized by staphylococcus antiserum. It is antigenically different from the *Streptococcus hemolyticus* fibrinolysin.

3. The staphylococcus anticoagulant has the same properties as the anticoagulant of other microorganisms: it inhibits coagulation of human and animal plasma continuously; it is produced in broths containing carbohydrates; it can be rendered ineffective by normal serum or spinal fluid; and it is not antigenic.

4. Staphylococcus lesions in man may be due to strains producing either the fibrinolysin or the anticoagulant. Both types of staphylococci may invade the blood stream.

5. Staphylococcus fibrinolysin as well as staphylococcus anticoagulant may be produced in staphylococcus infections in man.

6. Patients suffering from staphylococcus infections may develop a specific antifibrinolysin.

7. The plasma-clotting factor of staphylococci could be demonstrated in exudates of man.

8. The plasma-clotting factor of staphylococci may be inhibited by anticoagulants of staphylococci or of other micro-organisms without being destroyed.

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# INDEPENDENT VARIATION OF SEVERAL CHARACTERISTICS IN *S. MARCESCENS*

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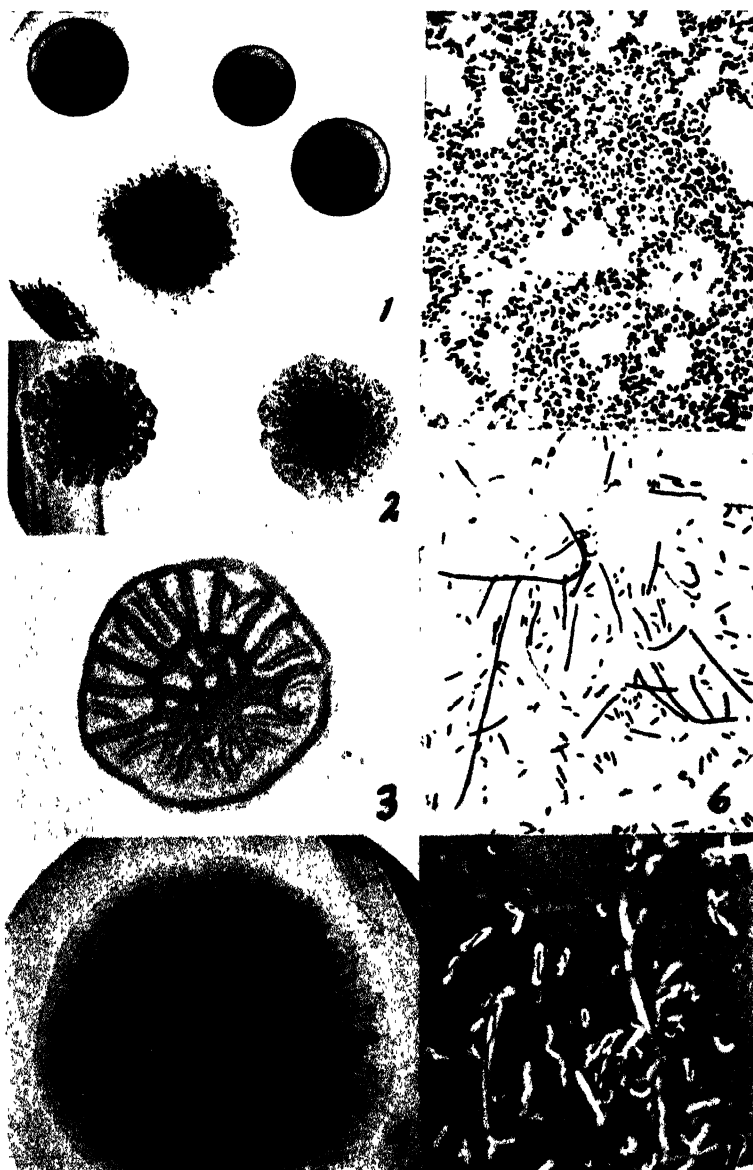
Variation in bacteria (S to R or R to S) is frequently accompanied by simultaneous change in several characteristics. Hadley's (1927) familiar list of characteristics generally associated with S and with R colonial forms summarises this relationship. There have been recorded, however, several instances of independent inheritance in individual characteristics. A study of variation and inheritance in *Serratia marcescens* suggests the presence of unit characters which may vary independently.

The wide variation in pigmentation of *S. marcescens* is well known, especially since the study made by Rettger and Sherrick (1911) twenty-five years ago. These authors do not appear to have observed variation in other characteristics and neither Breed and Breed (1924) in their monographic study of the genus nor Pederson and Breed (1928) in their detailed work on fermentation of this and related species make any reference to variation beyond noting the occurrence of non-pigmented strains and the difficulty of their classification.

In this study some six cultures from the National Collection of Type Cultures and a few recently isolated strains have been examined and two have been cultured in considerable detail over long periods.

## VARIETIES

Culture 2446 from the Collection of Type Cultures, described in their list as isolated from milk, Hygienisches Inst., Köln, has been particularly fruitful in the production of variants. Fifteen true-breeding types have been isolated, made up of the several



FIGS. 1 TO 7. PHOTOGRAPHS OF COLONIES AND ORGANISMS, *S. MARCESCENS*  
 Fig. 1. S and R colonies.  
 Fig. 2. R colonies.  
 Fig. 3. Medusoid colony.  
 Fig. 4. S colony showing radiating bands of red and white pigmentation.  
 Fig. 5. Organisms from a non-mucoid S colony.  
 Fig. 6. Organisms from non-mucoid R colonies.  
 Fig. 7. Organisms from a mucoid R colony.

combinations of three sets of characteristics: S and R and medusoid colonies; non-capsulated and capsulated forms; three colour varieties, red, orange-red and white.

*S colonies* on agar are low-convex to convex with regular entire margins and a perfectly smooth surface (fig. 1). Growth in broth is diffuse. The organisms, except in very old cultures, are always coccoid 0.3 to 0.6 in diameter or rods of the same diameter and up to 1.0 to 1.5 $\mu$  in length (fig. 5).

*R colonies* on agar are low-convex with erose to fimbriate margins and a rough or granular surface (fig. 2). Growth in broth is at first diffuse, like the S growth, but later becomes granular and tends to settle. In sharp contrast to the cells of S colonies, the R organisms are always rods from 1 to 8 or 10 $\mu$  in length and most R colonies contain many long filaments of 15 to 20 or occasionally 50 $\mu$  (fig. 6).

*Mucoid colonies* have been observed among both S and R types. The mucoid S colonies closely resemble the non-mucoid S except that the surface tends to glisten. The mucoid R colonies are indistinguishable, in appearance, from the non-mucoid R. On touching an S or R mucoid colony with a loop, or on dipping into a broth culture, mucilaginous threads may be drawn out to a length of several centimeters. The cells resemble those in the non-mucoid S or R colonies except for the presence of thick capsules (fig. 7). It is unusual to find R colonies with a mucoid structure but instances have been reported in other species as by McGaughey (1933) and Orr, Josephson, Baker and Reed (1934).

*Medusoid colonies* resemble medusa-head colonies described as occurring in several other species as in *Bacillus subtilis*, Soule (1928). On agar they are irregular mounds with reticulate to folded surfaces, rather than granular as in R colonies, and a thick irregular margin (fig. 3). The consistency of the colonies differs conspicuously from the mucoid and non-mucoid S and R in being tough and leathery. Growth in broth is at first diffuse and later granular like the R. The organisms resemble the mucoid R forms but tend to be irregularly curved. It seems probable, therefore, that the medusoid colony is the result of variation in the capsule structure of R forms.



*Colour varieties.* True-breeding red, orange-red and white colour varieties have been isolated from S, R, mucoid, non-mucoid and medusoid colonial forms. The red and orange-red pigments appear to be either closely related substances or modifications of the same substance. The colour contrast between the two is most evident when the organisms are grown on approximately neutral or acid agar, pH 7.5 to 6.0. On more alkaline media the

TABLE 1

*A list of variants isolated from seven cultures of S. marcescens*

CULTURE NUMBER	COLONIAL AND COLOUR VARIETIES	
2446	S non-mucoid	red, orange-red, white
	S mucoid	red, orange-red, white
	R non-mucoid	red, orange-red, white
	R mucoid	red, orange-red, white
	Medusoid	red, orange-red, white
2293	S non-mucoid	red, white
	R non-mucoid	red, orange-red, white
	R mucoid	red-orange, white
1377	S non-mucoid	red, white
	R non-mucoid	red, white
2302	S non-mucoid	red, white
	R non-mucoid	red, white
3804	S non-mucoid	red, white
2842	S non-mucoid	pink, white
	R non-mucoid	pink, white
air	S non-mucoid	red, pale pink, white

red appears less vivid and on agar of pH 8.0 to 8.5 it is impossible to distinguish the two. Transfers to neutral or acid media result in growths with the original sharply contrasting red and orange-red colours. When the two pigments are dissolved in alcohol the same contrasting red and orange-red colours are apparent but if two or three volumes of chloroform are added to the alcoholic solutions the colour contrast is lost.

Six of the above forms were isolated from the first series of plates made from the agar slope culture 2446 received from the Collection. The remaining nine types were recovered from later cultures. The fifteen have been maintained in pure culture for periods of six to twelve months. The idea that the original might not have been a pure culture has been rendered unlikely by the breeding history of the isolated types as described in the following section.

Six additional cultures of *S. marcescens* which have been examined in less detail have yielded two or more of the same sort of variants, as indicated in table 1.

#### VARIATION EXPERIMENTS

All of the types in the above list were tested for purity and stability by at least 12 serial single-colony isolations from agar plates before subjecting them to subsequent analysis. In the variation studies all cultures have been made in beef-infusion broth prepared according to Wright's (1929) method and on agar made from similar broth. Incubation has been at 18° or 37°. No other modification of the environment has been made.

##### *Variation in S. red type*

From a culture which for many generations, from single colony transplants had produced only S, non-mucoid, red types, six similar colonies were fished to 500-cc. flasks of broth and the cultures allowed to age at 18°. Plates were made at intervals from the ageing cultures. After two weeks a few orange-red and white colonies appeared on the plates and eventually the original red pigmented forms were largely replaced by these colour variants. After 24 days one of the six cultures developed a few mucoid colonies, some of which were red, some orange-red and some white. Two of the six cultures after 24 and 36 days produced a few R colonies, part of which were red, part orange-red and part white. The remaining cultures continued for the period of examination, 36 days, to produce only non-mucoid, S colonies.

Red, S forms, from the same source, were allowed to age for many days on agar plates at 18°. A very few colonies developed

R sectors or R marginal outgrowths from which pure R cultures were isolated. Similarly, colonies with orange or white marginal sectors frequently appeared, from which pure orange or non-pigmented cultures were readily obtained. This is exactly the end reached by Rettger and Sherrick (1911) by the more indirect method of gross selection from the least pigmented portions of agar slope cultures. It is also in agreement with many observations on pigmented species, especially with Hadley's (1927) results with *Pseudomonas aeruginosa* and Punkari and Henrici's (1933, 1935) results with a pigmented *Torula*.

A more extreme form of the same type of colony was frequently observed on ageing agar plates made from old broth cultures, colonies which were largely white but retained radiating bands of red or orange-red (fig. 4). This evidently results from the formation of several non-pigmented segments early in the history of the colony. More rapid growth of the white cells results in fan-shaped segments with a restriction of the pigmented cells to narrow bands until they are completely overgrown by the white cells at the margin of the colony. This again resembles *Torula* colonies described by Punkari and Henrici.

#### *Variation in 'S. mucoid, red type*

Four similar colonies were fished from a plate, made from a culture which for ten serial transfers from single colonies had produced only *S. mucoid*, red types, to 500-cc. broth flasks and allowed to age at 18° for 21 days. Plates made at intervals exhibited a few orange-red and many white colonies in all four cultures. Two of the cultures continued to produce only *S. mucoid* forms, one developed a few non-mucoid *S* forms and one a few mucoid *R* forms.

#### *Variation in R non-mucoid type*

Six broth tubes were inoculated from similar colonies of a culture which had produced only *R*, non-mucoid, red types for twelve serial cultures from single colonies. The broth cultures were incubated at 37° and serial transfers to broth made at 10- to 14-hour intervals when, judging by opacity, the cultures were

nearing the end of the logarithmic period of growth. Plate cultures made at intervals yielded a few orange-red and a few white colonies. Three cultures remained, as far as indicated by plating, entirely R and non-mucoid; one developed a few R mucoid forms and three developed a few S forms.

#### *Variation in R mucoid red type*

A similar variation has been observed in mucoid red types. In this instance out of five similar broth cultures, after eight rapid transfers, one remained unchanged, the other four developed orange-red and white variants; three produced S forms, mucoid, non-mucoid, red, orange-red, and white; one produced a few red, orange-red and white medusoid colonies.

#### *Variation in medusoid forms*

Medusoid colonies both red, orange-red and white, as noted in the last section, have very occasionally appeared in broth cultures of mucoid R strains. Once isolated these have proved to be among the most stable of the variations. Six similar red, medusoid colonies were fished to broth tubes and transferred serially for twenty 10- to 14-hour generations at 37°. Plate cultures made at intervals up to the last generation developed only medusoid colonies but five to twenty per cent were orange-red or white. When plate cultures from the latter broth generations were allowed to age for two to three weeks at 18° a very few colonies developed marginal sectors of mucoid or non-mucoid R. Fishings from these sectors yielded, eventually, true-breeding-mucoid and non-mucoid R cultures, some red, some white.

#### *Variation in pigmentation*

In the preceding paragraphs it has been shown that red, orange-red and white varieties have been isolated from S, R and medusoid colonies and from capsulated and non-capsulated types. These have been maintained as true-breeding colour varieties for many generations. Variation in pigmentation has been observed to occur in one direction only. Change occurs readily from red to orange-red or white, orange-red to white, or in another culture

from red to pale pink or white, pale pink to white, but in neither case has a single instance of variation in the reverse direction been observed. This is in agreement with Hadley's finding in the case of *P. aeruginosa* (1927).

#### ANTIGENIC STRUCTURE

Antigenically, *S. marcescens* is apparently not a homogeneous species. Anti-sera prepared in rabbits against both S and R forms of two cultures agglutinated, in large floccules, S organisms from the homologous and from four additional cultures but failed to agglutinate S organisms from two other cultures in sufficient serum dilution to indicate significant antigenic relationship. This suggests that there may be agglutination groups within the species but the matter has not been further investigated.

TABLE 2

*Agglutination reactions of six cultures of S. marcescens in the S, red, non-mucoid form. Floccular agglutination*

ANTI-SERA	S ORGANISMS					
	Culture 2446	Culture 2293	Culture 1377	Culture 2302	Culture 3804	Culture 2842
2446, S, red, non-M.	1-2560	1-1280	1-640	1-1280	1-20	1-20
2446, R, red, non-M.	1-20480	1-20480	1-20480	1-20480	1-20	1-20
2293, S, red, non-M.	1-10240	1-10240	1-10240	1-10240	1-20	1-20
2293, R, red, non-M.	1-20480	1-1240	1-20480	1-20480	1-20	1-20

Within a single strain on the other hand variation in pigmentation has no influence upon the flagellar antigen. As shown in table 2, anti-sera for red pigmented S forms, cultures 2446 and 2293, produce, at the same maximum dilution, floccular agglutination of red and white S, red and white R organisms. Similarly, anti-sera for white forms agglutinate at the same maximum dilution red and white S, red and white R organisms. The problem of the classification of non-pigmented *S. marcescens* raised by Pedersen and Breed (1928), it appears, might be facilitated by agglutination studies.

In the same manner variation of non-mucoid to mucoid or medusoid forms does not appreciably affect the flagellar antigen. Where anti-serum for either the non-mucoid or mucoid type is

brought into contact with non-mucoid, mucoid or medusoid organisms, in series of agglutination tubes, arranged so that the serum dilution is doubled in each successive tube, the maximum serum dilution for floccular agglutination of mucoid and medusoid organisms is ordinarily one tube less than the dilution which affects agglutination of non-mucoid organisms. This relatively small difference seems more likely to result from alteration in the physical character of the mucoid organisms than from variation in the flagellar antigen.

Variation in colony structure from S to R resulted in complete loss of somatic antigen but had no influence on the flagellar agglutination. There was, however, some difficulty in removing flagellar antigen. Heating suspensions of S or R organisms to 75°

TABLE 3

*Agglutination reactions with pigmented and non-pigmented S. marcescens*

ORGANISMS	ANTI-SERUM			
	2446, S, red	2446, S, white	2293, S, red	2293, S, white
2446, S, red	1-2560	1-2560	1-10240	1-1280
2446, S, white	1-2560	1-2560	1-10240	1-1280
2446, R, red	1-5120	1-2560	1-10240	1-1280
2446, R, white	1-5120	1-2560		

for an hour, which ordinarily destroys or inhibits the H antigen, or extracting with absolute alcohol at 50°, the method used by White (1927), only served to lower the agglutination titre from, for instance, 1-5120 to 1-1280 or 1-640 but did not alter the form of flocculation of either S or R organisms. White (1928), however, found that the H antigen of certain R *Salmonella* types was resistant to this treatment but was inactivated by a further extraction with chloroform. As indicated in table 3, S organisms extracted with alcohol-chloroform gave clear cut fine granular or O agglutination with both anti-S and anti-R serum at approximately one fourth the maximum serum dilution which produced characteristic large floccules or H agglutination with the untreated S organisms. R organisms, on the other hand, after alcohol-chloroform extraction fail to agglutinate with either anti-S

or anti-R serum. As in many other species, the flagellated S organisms contain both H and O antigens. In S to R variation the H antigen is retained, the O antigen is lost.

#### ASSOCIATED AND INDEPENDENT FACTORS IN INHERITANCE

In this study the inheritance of five sets of characteristics has been followed. In the preceding description of variants and their breeding history it has been shown that colony structure, cell form and specific somatic agglutinin are always associated: S colonies are composed of short rods and coccoid cells containing specific somatic antigen, while the R colonies are composed of long rods and filaments without the specific somatic antigen.

TABLE 4

*Agglutination reactions with S and R organisms from young broth cultures and organisms extracted with alcohol-chloroform*

ORGANISMS	ANTI-SERUM			
	2446 S	2446 R	2293 S	2293 R
2446, S, motile	1-2560 fl.	1-20480 fl.	1-1280 fl.	1-40960 fl.
2446, S, alc.-chl.	1-640 g.	1-640 g.	1-320 g.	1-320 g.
2446, R, motile	1-5120 fl.	1-40960 fl.	1-1280 fl.	1-40960 fl.
2446, R, alc.-chl.	0 ..	0	0	0

fl. = floccular. g. = granular agglutination.

Where variation occurs from S to R or in the reverse direction the associated characteristics vary synchronously. This may arise from an inseparable linkage of the inheritance factors or these three characteristics may result from the transmission of a single gene. What is measured as somatic antigen may, for instance, determine the cell form and cell form may determine the colony topography.

It has been shown, however, that capsulated cells with the resulting mucoid structure of colonies occur in both S and R forms. Variation may proceed in the direction of either gain or loss in capsulation and this variation may occur irrespective of variation in S or R colony form. It is evident, therefore, that the factor for the inheritance of mucoidness or its absence is independent of the factor or factors for colony structure.

It has also been shown that variation in pigmentation from red to orange-red or white or from orange-red to white, occurs regardless of variation in colony structure or capsulation of the cells. Colour factors must therefore be inherited independent of factors for colony structure or for capsulation.

Independent variation in three sets of characteristics does not appear to be consistent with any theory of cyclic change in the organisms. It seems more likely that, in these cases at least, the inheritance of individual characteristics has been subject to some irregularity. A mechanical hypothesis was recently suggested (Reed 1933) depending upon assumed irregularities in cell division and the distribution of multiple genes for a single characteristic. However, multiple gene theories have not proven to be workable in higher forms where more data are available; indeed Muller (1932) has shown that the results produced are not proportional to the "concentration" of genes. It therefore seems more in line with current genetical opinion (Muller 1932), to assume that the independent variation in several characteristics, as demonstrated in this species, is the result of change in individual genes or "gene mutation."

#### CONCLUSIONS

1. It has been shown that in the variation of *Serratia marcescens* colony structure, cell form and somatic antigen are inseparably associated.

2. Variation in capsulation of the cells and in pigmentation may occur, independent of variation in colony structure and associated characteristics.

3. The suggestion is made that this independent variation in several characteristics, is more likely to result from gene mutations than from a cyclic change in the organism.

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# A NEW METHOD FOR THE EVALUATION OF GERMICIDAL SUBSTANCES

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The usual procedure in testing the germicidal strength of a compound is to note its effect on one or more species of bacteria and to compare the results with those obtained from phenol. A value known as the phenol coefficient may be calculated from the results of such a test.

The phenol coefficient is defined as the killing power of a germicide, towards a specified organism, as compared to that of phenol. If the organism, *Eberthella typhosa* (*B. typhosus*) is used as the test organism the result will be an *Eberthella-typhosa* phenol coefficient, etc. The phenol coefficients will vary, naturally, with the test organism employed.

Several methods are used to determine the phenol coefficient. That proposed by Reddish (1928), and recommended by the American Public Health Association, was followed by us in every case.

Briefly, the phenol coefficient is determined by inoculating various dilutions of the germicide in sterile distilled water with the test organism. After an interval of five minutes a standardized loopful<sup>1</sup> is removed from each dilution and transferred to broth. This is repeated after ten minutes and again after fifteen minutes. The tubes are examined for turbidity after 48 hours. The same procedure is followed for phenol. The phenol coefficient is determined by dividing the greatest dilution of disinfectant capable of killing the test organism in 10 minutes but not in five minutes by the phenol dilution found to effect this result.

<sup>1</sup> A 4 mm. loop, outside diameter.

Various factors must be controlled in order that the results shall be of value. These include: species of organism, viability of the organism, temperature of incubation, proportion of culture to disinfectant, size of the inoculation loop, and composition of the culture medium.

It is customary to rate disinfectants on the basis of their phenol coefficients. The method may be justifiable if the germicides are to be employed on external skin surfaces or in connection with the sterilization of non-living material. On the other hand, if disinfectants are internally administered or used on mucous surfaces, the above method presents serious objections. A germicide possessing a high phenol coefficient is usually preferred to one less potent. There would be no advantage in using a compound with a coefficient of 10 on mucous surfaces if it possessed ten times the toxicity of phenol. Phenol could be employed to equal advantage, with probably a considerable saving in the cost of the disinfectant.

It is believed that a more accurate method of rating those germicides, recommended for internal administration or for use on mucous membranes, would be to test them for their effect on the growth of living embryonic tissue as well as for their ability to kill bacteria. A number, known as the toxicity index is determined by dividing the highest dilution required to prevent the growth of embryonic chick heart tissue during 48 hours by the highest dilution required to kill the test organism after an exposure of 10 minutes.

For the preparation of tissue cultures it is necessary to have: (1) Plasma, (2) Tyrode solution, (3) tissue fragments and, (4) diluted embryonic fluid.

1. *Plasma.* Guinea-pig plasma is satisfactory and easily obtained. Ten cubic centimeters of blood are mixed with 1.0 cc. of a 1:1000 heparin solution. This prevents rapid coagulation of the plasma. The blood is centrifuged, the plasma removed and refrigerated until used.

2. *Tyrode solution.* This is a physiological salt solution containing 0.1 per cent glucose. It is used probably more than any other physiological salt solution for tissue culture work.

3. *Tissue fragments.* Chick hearts removed from 9-day-old embryos are used in pieces from 0.5 to 1.0 mm. in diameter. The size should be as uniform as possible, never larger than 1.0 mm. in diameter.

4. *Dilute embryonic fluid.* This is prepared by mincing chick embryos in a tissue grinder and diluting with 5 parts of Tyrode solution. The suspension is then centrifuged and the clear supernatant fluid removed. The embryonic fluid serves as food for the tissues.

The various dilutions of the germicide under examination are made in the embryonic fluid.

The tissue cultures are prepared as follows: The fragments are embedded in guinea-pig plasma diluted with three parts of Tyrode solution in Carrel flasks. The plasma coagulates shortly after the addition of the tissue. The fibrin matrix holds the fragments. The coagulated plasma is then covered with a layer of embryonic fluid containing a known concentration of the germicide. The flasks are capped to prevent evaporation and incubated at 38°C.

The plasma, Tyrode solution and embryonic extract are carefully measured so that the final concentration of germicide in each flask is known.

The flasks are examined for tissue growth after an incubation period of 48 hours.<sup>2</sup> The highest dilution of the germicide showing no growth of the tissue is taken for the calculation of the toxicity index. The following example will show the method for determining the index. The highest dilution of disinfectant preventing growth of tissue in 48 hours is 1:5000. The highest dilution of the chemical showing no growth of the test organism, *Staphylococcus aureus*, after 10 minutes' exposure,<sup>3</sup> is 1:2500. The toxicity index is calculated by dividing 5000 by 2500. The result, 2.0, means that the germicide is twice as toxic for the tissues

<sup>2</sup> A period of 24 to 48 hours is required in order to determine if the tissue fragments show proliferation.

<sup>3</sup> The exposure time of the organisms to germicide was limited to 10 minutes because it is not believed that a chemical in contact with a mucous membrane or the cut surfaces of an incision will remain effective for a longer period of time.



TABLE 1

*Toxicity of germicides to chick heart tissue and Staphylococcus aureus*

GERMICIDE	HIGHEST DILUTION SHOWING NO TISSUE GROWTH = A	HIGHEST DILUTION SHOWING NO GROWTH OF STAPHYLOCOCCUS AUREUS = B	TOXICITY INDEX = A/B	STAPHYLOCOCCUS AUREUS PHENOL COEFFICIENT
Iodine. . . . .	1:1,800	1:20,000	0 09	308
Iodine trichloride. . . . .	1:2,400	1:6,000	0 40	92
Mercuric chloride. . . . .	1:45,000	1:16,000	2 8	246
Hexylresorcinol. . . . .	1:21,000	1:7,000	3 0	108
Metaphen. . . . .	1:76,000	1:6,000	12.7	92
Phenol. . . . .	1:840	1:65	12.9	
Potassium mercuric iodide . . . .	1:12,000	1:900	13 3	13 8
Merthiolate . . . . .	1:176,400	1:5,000	35 3	70
Mercurochrome. . . . .	1:10,500	1:40	262 0	0 6

TABLE 2

*Toxicity of germicides to chick heart tissue and E. typhosa*

GERMICIDE	HIGHEST DILUTION SHOWING NO TISSUE GROWTH = A	HIGHEST DILUTION SHOWING NO GROWTH OF E. TYPHOSA = B	TOXICITY INDEX = A/B	E. TYPHOSA PHENOL COEFFICIENT
Iodine. . . . .	1:1,800	1:24,000	0 08	240
Potassium mercuric iodide . . . . .	1:12,000	1:110,000	0 11	1,100
Mercuric chloride . . . . .	1:45,000	1:180,000	0 25	1,800
Iodine trichloride. . . . .	1:2,400	1:8,500	0 28	85
Metaphen. . . . .	1:76,000	1:90,000	0 84	900
Hexylresorcinol. . . . .	1:21,000	1:7,500	2 8	75
Phenol. . . . .	1:840	1:100	8 4	
Merthiolate. . . . .	1:176,000	1:5,000	35	50
Mercurochrome. . . . .	1:10,500	1:300	35	3

## DISCUSSION

The determination of the phenol coefficient is not an accurate basis for evaluation of disinfectants employed under all conditions. The method is practical for rating disinfectants to be employed for the sterilization of non-living material. On the other hand the method fails to take into account the toxicity of germicides for living tissues when used internally or on mucous surfaces. A more valuable expression would be one based on a

combination of the killing power of the disinfectant for bacteria with its toxic action toward living embryonic tissue.

This method gives a figure which is known as the toxicity index. The toxicity index is defined as the ratio of the highest dilution of disinfectant required to prevent the growth of embryonic chick heart tissue during 48 hours to the dilution required to kill a given test organism in 10 minutes.

The toxicity indices are not always proportional to the phenol coefficients. Iodine trichloride and metaphen have the same phenol coefficient yet metaphen is about 32 times more toxic to embryonic chick heart tissue than the iodine compound (table 1). As a germicide merthiolate is over 100 times more potent than mercurochrome but only about one-eighth as toxic to tissue.

An important conclusion is that the older and simpler germicides rate considerably higher than the newer and more complex compounds. Iodine is one of the oldest germicides known. It is usually employed as tincture of iodine, an alcoholic solution, which is quite irritating, due to the presence of the alcohol. Because of this irritating property the alcoholic solution is gradually losing favor. In aqueous solution, however, the iodine is non-irritating and relatively non-toxic to tissue. It is the most ideal of any of the compounds studied when tested by the tissue culture technique.

Of the newer organic preparations examined hexylresorcinol gave the lowest toxicity index on *Staphylococcus aureus* (gram-positive), while metaphen gave the lowest figure when tested on *Eberthella typhosa* (gram-negative).

The above germicides are being retested in an attempt to control some of the more obvious variables. Some of these are, (1) temperature, (2) time of action of chemical on bacteria and tissue, and (3) presence of organic matter.

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# A STUDY OF MENINGOCOCCI RECOVERED IN THE UNITED STATES SINCE 1930

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The first recorded outbreak of meningococcus meningitis in the United States was in Medford, Massachusetts, in 1806 (Clymer, 1872). Records since that time indicate a periodic return to epidemic proportions at intervals of approximately ten years. Hedrich (1931) has studied reported epidemics and has concluded that the interval falls oftenest between 6 and 12 years in length, though some localities may miss the wave, showing an interval of 20 to 25 years.

The meningococcus was recognized as the cause of the disease by Weichselbaum (1887) but serological differences were not noted until the observations of Dopter (1909) and workable classifications were not available until the intensive work done during the World War by Gordon and Murray (1915) and Nicolle, Debains and Jouan (1918). Hence, the relation between the epidemic waves of meningitis and the predominance of various serological groups of meningococci could be studied only during the last twenty years, and it will not be until more decades have passed that it can be known whether or not there is regularity in the serological changes which occur, and what the epidemiological significance of such changes may be.

This periodicity in return of epidemic conditions can be seen in table 1, which gives the total number of cases of meningococcus meningitis reported in the United States since 1915.<sup>1</sup> These figures are not absolutely accurate since the number of States reporting is not the same for all years. The number of strains of

<sup>1</sup> Information obtained from the Division of Sanitary Reports and Statistics, United States Public Health Service.

meningococci studied at the National Institute of Health for those years is also listed in this table. Peaks in incidence of cases occurred in 1918, 1929, and 1936. Thus far (March 1, 1937) there has not been an increased number of cases over 1936. The number of cultures of meningococci sent into the National Institute of Health for study during the endemic years has been small.

Meningococci that were received during the years 1918-19 and 1921 and 1922 were studied and reported by Evans (1922) and

TABLE 1

*Number of cases of meningococcus meningitis reported in the United States for the years 1915-1936, inclusive, and number of strains studied at the National Institute of Health during those years*

YEAR	NUMBER OF STATES INCLUDED	TOTAL CASES	STRAINS STUDIED AT NATIONAL INSTITUTE OF HEALTH	YEAR	NUMBER OF STATES INCLUDED	TOTAL CASES	STRAINS STUDIED AT NATIONAL INSTITUTE OF HEALTH
1915	?	1,403		1926	35	1,700	
1916	?	1,748		1927	38	3,001	4
1917	30(?)	4,705		1928	40	4,996	57
1918	30(?)	5,749	63	1929	46	10,551	89
1919	30(?)	2,417		1930	44	8,384	82
1920	30	2,258		1931	41	5,518	26
1921	30	2,002	27	1932	41	3,102	19
1922	30	1,527	14	1933	44	2,913	7
1923	35	1,506		1934	45	2,500	10
1924	35	1,223		1935	43	5,736	91
1925	35	1,253		1936	45	6,528	299

those during the years 1927-30 by Branham, Taft, and Carlin (1931). The present report discusses especially those strains studied since 1930.

In all of the studies referred to above, the meningococci were "typed" according to the Gordon-Murray (1915) classification. In 1918-19 and 1921-22, Evans did this typing on the basis of standard type cultures received through the Rockefeller Institute. In our own studies of 1927-30 a preliminary typing was done on the basis of these same strains. It is well known that meningococci tend to "spread" antigenically after more or less long periods

of laboratory maintenance and become less type specific, so Dr. Gordon's offer of the dried antigens which he had prepared from his original type strains in 1915 was welcomed. The typing of our 1927-30 strains was, therefore, checked with type sera made with Dr. Gordon's original preparations.

Intensive studies were done with these 1927-30 strains and an analysis of their serologic patterns made in comparison with the original Gordon-Murray type preparations. On the basis of this study, new standard strains were chosen which seemed more closely to resemble the original type strains than any others. At first these were used in conjunction with the older ones, but as these newer strains proved more satisfactory than the old, they have been employed as a basis for "typing" ever since.

Since January, 1931, we have typed approximately 500 strains of meningococci. These represent both endemic and epidemic years; the number received in each of these years is shown in table 1.

When these cultures were first received they were examined for purity, their cultural characteristics and fermentation reactions were observed, and they were typed.

Cultural characteristics were typical. The enormous colonies noted frequently during 1928-29 were not observed in any of the strains that came to us during these later years.

Heretofore it has been our observation that all meningococci have ultimately produced acid in glucose and maltose media. In the present series there have been four strains with which fermentation of maltose has not been observed (one of Type I and three of Type II), and two strains which have not been proved to ferment glucose (both type I). Acid production is often both slight and very transient and can be easily overlooked. One strain began to ferment glucose after a year in the laboratory, so it is possible that these negative strains referred to above will ultimately ferment both glucose and maltose regularly.

Sera for typing were prepared by injection of young rabbits with the standard type strains; viz., 331 (I), 173 (II), 302 (III), and 158 (IV). The cultures were grown for 18 hours on 0.5 per cent glucose agar slants, suspended in 0.85 per cent saline, diluted

to approximately 1,000,000,000 meningococci per cc., and injected immediately. It has been found by workers in our laboratory<sup>2</sup> that rabbits respond better to Types I and III if the first injection is given intracutaneously. Thus, an initial intracutaneous injection of about 400,000,000 meningococci, followed by four intravenous injections on successive days, usually gave a satisfactory typing serum. With Types II and IV it was desirable to repeat the series after a week of rest. A brief period of immunization usually resulted in sera which were more type specific than those obtained by longer immunization, though the agglutinin titer was somewhat less high.

TABLE 2

*Type distribution of strains of meningococci from active cases since 1930*

YEAR	I	I-III	III	I AND III	II	IV	NOT TYPED	TOTAL STRAINS
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
1931	16 0	16 0	64 0	96 0	4 0			25
1932	31 0		37 0	68 0	32 0			19
1933	28 5	14 2	14 2	57 0	43 0			7
1934	20 0		40 0	60 0	40 0			10
1935	29 6	27 5	27 5	84 4	13 2		2 2	91
1936	44 0	15 1	30 0	89 1	8 7		2 7	257
All years....	37 6	28 8	19 5	86 0	11 7		2 2	409

Typing was by simple agglutination, the technique used being the same as that described by the authors in earlier reports (1931). Absorption of agglutinins was not done. It has been shown by one of us (Branham, 1932) that Types I and III are so closely related that even absorption of agglutinins will not "type" some strains. Scherp and Rake (1933, 1934, 1936) have shown the specific polysaccharide of these two types to be the same. For routine purposes it seems impracticable to spend time in trying to separate all strains of this I-III group into the two types I and III. In these present studies we have designated strains as Type I or Type III if they seemed definitely to be one or the

<sup>2</sup> Observations made by Fabst and confirmed by Pittman.

other; but in cases where the overlapping was great we have designated them as belonging to group I-III. In table 2, the type distribution is shown for each of the years since 1930. The results are expressed in percentage, and the total number of strains studied during each year is also given. No "carrier

TABLE 3  
*Type II meningococci from active cases since 1930*

YEAR	ACTUAL NUMBER OF TYPE II STRAINS	(ALL TYPES) TOTAL STRAINS
1931	1	25
1932	6	19
1933	3	7
1934	4	10
1935	12	91
1936	22	257

TABLE 4  
*Serological grouping of all strains reported at the National Institute of Health since 1918*

YEAR	NUMBER OF STRAINS	I-III	II	IV	N. FLAVES- CENS	NOT IN THESE TYPES
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1918-19	128	58.6	25.8	2.3		13.3
1921	16	31.3	18.7	6.3		43.7
1922	15	6.7		13.3		80.0
1928-30	235	81.6	5.5	7.6	5.9	
1931	25	96.0	4.0			
1932	19	68.0	32.0			
1933	7	57.0	43.0			
1934	10	60.0	40.0			
1935	91	84.4	13.2			2.2
1936	257	89.1	8.7			2.7

strains" are included in this table, but only strains from spinal fluid or blood.

The year 1931 may be considered the end of the epidemic period which began in 1928, and the year 1935 represents a return of epidemic conditions. During the endemic years of 1932, 1933, and 1934 the percentage of Type II strains in cases of

meningitis studied rose from 4 per cent in 1931 to 32.0 per cent in 1932, and 43.0 per cent and 40.0 per cent in 1932 and 1933, respectively, falling to 13.2 per cent when epidemic conditions returned in 1935. The actual number of cases studied during the endemic years was very small, but the fact that so many of them proved to be of Type II seems to be not without significance. Table 3 shows the actual number of Type II strains identified during these years. The slight increase in number during 1935 and 1936 seems insignificant when compared with the increase in the total number of strains studied.

Table 4 shows the type distribution of all meningococci reported from the National Institute of Health since 1918. Reference to this table indicates that Type II, which was abundant in the 1918-19 epidemics during the World War, has played a very insignificant rôle in the United States during the last two epidemic waves, and that Type IV seems to have dropped out of the picture altogether. The epidemic conditions occurring during these years have been due almost entirely to a very great increase in the I-III group of meningococci.

In table 2 the total number of strains in the I and III group is expressed as a whole in the fifth column. These are subdivided into I, I-III, and III, in columns two, three, and four. This analysis shows that the Type III strains were predominant at the end of the epidemic of 1928-31, comprising 64 per cent of all studied. During 1935, the first year of the latest epidemic wave, there was approximately an equal number of I strains, III strains, and strains which we designate as I-III because they were agglutinated equally well by both I and III type serums. During 1936 there was a steady shift in serological pattern toward Type I, and this trend has been even more marked among the meningococcus cultures received to date during the first two months of 1937.<sup>2</sup>

Of the total strains reported in this paper, only 20 are known definitely to have been isolated from blood; 16 of these fell into

<sup>2</sup> Of 34 strains studied in these last two months, 20 (or nearly 60 per cent) have been of Type I; 5 (or 13.7 per cent) have been of Type III; 6 (or 17.6 per cent) have been of I-III; and 3 (or 8.8 per cent) of Type II.

the I-III group, and 4 were of Type II. These Type II strains comprised 20 per cent of the strains from septicemic cases without meningeal involvement, which is a high proportion when the low number of total Type II strains is considered.

It has seemed best to consider the 42 carrier strains separately, since most of these were obtained under circumstances which made them not comparable to those from active cases. All except one of these 42 nasopharyngeal strains were received by us during the spring of 1936. Five were found among the contacts of active cases of meningitis in a CCC camp. Thirty-four were isolated during a carrier survey made in the same camp a few days later. The other three were from people in scattered localities who were in contact with active cases. The results of typing these strains may be seen in table 5. The predominance of Type II strains among these carriers gives support to the impression gained by

TABLE 5  
*Types of strains of meningococci found in carriers*

I	III	II	NOT IN KNOWN TYPES	TOTAL NUMBER
9 (21.4%)	3 (7.1%)	24 (57%)	6 (14%)	42

Gordon and Murray (1915) and by Scott (1918) during the World War that Type II was more common among carriers than among active cases. A similar preponderance of Type II among carrier strains has been found by Rake (1934). The 24 Type II strains reported in table 5 were all found among the 35 strains obtained in the carrier survey made in the CCC camp; all strains obtained from persons in contact with active cases were of the same type as those isolated from the patients, none of whom were infected with Type II organisms.

#### DISCUSSION AND SUMMARY

Records of the occurrence of meningococcus meningitis in the United States since the first reported outbreak in 1806 indicate that this disease has a tendency to reach a peak in incidence at intervals which approximate 10 years.



A correlation of these increases in incidence with the serological classification of the meningococci involved has been possible only during the last 20 years or so—a period which involves three recrudescences in which the disease reached epidemic proportions.

Serological studies of the meningococci received at the National Institute of Health have been reported for the periods 1918–22 and 1928–30. In the present report those strains received since 1930 are described.

During the years 1931–36 four hundred and fifty-one strains of meningococci have been typed according to the Gordon-Murray classification. As found in previous studies, Type I and Type III are so closely related that it is often unprofitable to attempt to separate them; every possible intergradation seems to occur. Many strains have been definitely I or definitely III, but others have been agglutinated equally by both I and III sera. Absorption of agglutinins has not been done with these strains except for purposes of special research. For routine work this technique has seemed to be a waste of time. Such strains have been classified as I–III. In most of the tables included in this report we have grouped all I and III strains together as I–III, but in table 2 this group has been resolved into I, III, and I–III, in order that the present trend in changing serological pattern of prevalent strains from Type III (1931) to Type I (1936) may be seen.

For practical purposes we may consider that we have met two groups of meningococci during the last decade; viz., groups I–III and II. We have encountered no strain of IV or of *Neisseria flavescens*, both of which played an important rôle in Chicago in 1928. Study of the five tables included in this report will show that the I–III group of meningococci has been responsible for most of the active epidemic cases. Type II has been more frequently found in carriers, and its relative incidence in cases has been greater during the endemic years.

Whether Type II is to be considered as generally less virulent than the meningococci of the I–III group, or whether this minor rôle which it seems to play in our present epidemics is merely characteristic of this last decade, can only be determined in the future.

## CONCLUSIONS

In this report, 409 strains of meningococci from spinal fluid and blood of persons with meningococcus infection, and 42 strains from carriers, received since 1930, have been "typed" according to the classification of Gordon and Murray. Of the strains from active cases, 352 (or 86.0 per cent) fell into the I-III group (including types I and III, and strains agglutinated equally well by both serums); 48 (or 11.7 per cent) were of Type II.

Of 42 carrier strains, 24 (or 57.0 per cent) were of Type II, and 12 (or 28.5 per cent) were of group I-III. Of these twelve I-III strains, 8 were from individuals who were in immediate contact with cases due to that type of meningococcus.

Apparently Type II has been relatively unimportant as a cause of epidemic meningitis in the United States during the last decade. On the other hand, the prevalence of Type II strains among the carriers studied, and the relatively greater occurrence of this type in active cases during the endemic years of 1932 to 1935, throws some light on the possible significance of this group.

It is impossible to judge at this time whether the epidemiological differences noted between groups I-III and II are a rule, or if they are an accident of this decade.

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# RELATIONSHIPS BETWEEN STAPHYLOCOCCI AND BACILLI BELONGING TO THE SUBTILIS GROUP AS SHOWN BY BACTERIOPHAGE ABSORPTION<sup>1</sup>

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Experimental evidence has established the fact that with few exceptions bacteriophages are absorbed by susceptible bacteria, either living or dead, (d'Herelle, 1920; Bail and Okuda, 1923; Prausnitz and Firle, 1924; Burnet, 1930). Exceptions have been noted by Flu, 1923; Gohs and Jacobson, 1927; Levine and Frisch, 1934; Burnet, 1934; and ourselves. The studies of Burnet (1929-34) have demonstrated that absorption of phage is largely determined by the nature of the surface antigens of the bacterial cell. This concept has been strengthened by recent investigations dealing with bacterial extracts, first reported by Levine and Frisch (1933-34). Extracts prepared from susceptible bacteria have the power of inactivating phage, while those of lyso-resistant strains lack this property. The majority of reports dealing with phage absorption or inactivation have resulted from evidence obtained by a study of the enteric group of bacteria and their phages.

Staphylococcus phages behave in a similar manner toward susceptible cultures and extracts derived from them, (Rakieten, *et al.*, 1936). However, toward such agents as serum and white blood cells staphylococcus phages in general differ from those attacking the Gram-negative organisms in that they are rendered inactive to a varying degree. This so-called non-specific absorbing power of body fluids and exudates for staphylococcus phages may be modified to a point of practical extinction by a method

<sup>1</sup> Presented in part at the annual meeting of the Society of American Bacteriologists, December 1936, New York City.

entailing adaptation of staphylococcus phage to serum (d'Herelle and Rakieten, 1935). Staphylococcus phages also differ from coli and dysentery phages in that they may be absorbed by organisms other than susceptible cocci, namely bacteria belonging to the subtilis group. Such organisms as far as can be determined are not affected by their contact with staphylococcus phages; the phage corpuscular count, however, is diminished enormously. No absorbing action, on the part of these Gram-positive bacilli, was ever demonstrated against coli, dysentery, proteus, or streptococcus phages. The present study deals with the staphylococcus-phage-absorbing ability of certain members of the subtilis group and a discussion of the mechanism responsible.

In 1933 a tube of polyvalent staphylococcus phage became contaminated while standing on the laboratory table. On refiltering, the previously active phage was without lytic or plaque-producing power. The contaminant was an actively motile, large, Gram-positive rod. In Savita broth a slight turbidity and a firm wrinkled pellicle develops in twenty-four hours at 35°C. The same characteristics are noted when the organism is allowed to grow at room temperature. Gelatin is liquefied actively, and hemolysis is produced on blood agar plates. On Savita agar the organisms produce dark brown to black colonies, and over a varying period of time the pigment diffuses into the agar, coloring it brown. The colonies are flat, dry, and wrinkled, though, very infrequently there have developed black, raised, and mucoid colonies. Associated with this temporary change there has been a decrease in the phage-absorbing quality of the organism. Central spores may be observed when growth occurs after twenty-four hours at incubator temperature in meat-extract peptone broth and on Savita agar, but in Savita broth spores appear only after several days incubation. This particular strain of *B. subtilis* (*niger*) hereafter designated as strain C3 has been studied with regard to its staphylococcus-phage-absorbing ability. While we have isolated 27 other strains belonging to the subtilis group and examined them for phage absorbing power, to a large extent our interest has centered on culture C3. Experiments

with the few other subtilis strains that possess this character lead us to believe that a mechanism similar to that of strain C3 is responsible for this action.

#### EXPERIMENTAL

##### *Absorption of staphylococcus phages by strain C3*

To tubes of broth (approximately 10 cc.) 0.1 cc. of a twenty-four-hour culture of strain C3 and 0.25 cc. of bacteriophage were added. The tubes along with proper controls were incubated at 35°C. At frequent intervals tubes containing culture and phage, as well as tubes containing phage alone were filtered through L5 Chamberland candles, in an effort to find out at what period during the growth of the culture phage absorption occurs. Portions of the filtrate were layered over segments of agar plates that previously had been seeded with a susceptible culture of staphylococcus. After proper incubation the plates were counted for plaques. The protocol in table 1 summarizes the results obtained when strain C3 was tested for its phage-absorbing ability when in contact with three polyvalent staphylococcus phages.

In each instance it may be observed that there is a drop in corpuscular count of staphylococcus phage after addition to a tube containing strain C3. As bacterial multiplication begins, certainly within the third hour, a continued decrease in phage elements takes place until, within a relatively short period after growth may be observed macroscopically, the phage titer drops to such an extent that in the dilutions noted no plaques are demonstrable. Under the same conditions, however, control tubes of phage alone show little if any fluctuation in titer. Repeatedly, filtrates of phage and culture mixtures were tested for evidence of phage on several other susceptible cultures of staphylococci by the cross test (Asheshov, 1933), without positive indication of lysis.

Since it appeared that a relation existed between bacterial cell numbers and reduction in bacteriophage corpuscle count (controls showed that this was not associated with a significant change in pH) one should be able to demonstrate this almost immediately, providing sufficient bacterial cells are added to a bacterio-

phage. The following experiment was carried out to test this assumption. A twenty-four-hour broth culture of strain C3 was centrifuged at high speed for thirty minutes. The supernatant fluid (7.5 cc.) was removed and the sedimented bacteria resuspended in the same volume of Savita broth. To this was added 0.25 cc. of staphylococcus bacteriophage M13. Similar amounts of phage were added to the supernatant and a control tube of broth. All tubes were shaken vigorously for a few minutes and then filtered through L5 Chamberland candles. A dilution of  $10^{-5}$  of each filtrate was then overlayed on an agar-plate culture

TABLE 1

*Plaque count after layering a dilution of  $10^{-5}$  of each filtrate on susceptible culture Staphylococcus aureus Vnt.*

	IMMEDIATELY	30 MINUTES	1 HOUR	3 HOURS	5 HOURS	24 HOURS
C3 plus Bp. M13. . . . .	175	201	256	112	6	0
Control Bp. M13. . . . .	500 (cfl.)	cfl.	cfl.	cfl.	cfl.	cfl.
C3 plus Bp. P.G. . . . .	197	142	211	17	0	0
Control Bp. P.G. . . . .	414	449	394	N.T.	401	408
C3 plus Bp. Lrk. . . . .	206	115	111	185	80	0
Control Bp. Lrk. . . . .	cfl.	458	417	489	cfl.	cfl.
C3 control (growth in tube-macroscopic) . .	—	—	—	±	+±	+++

cfl. = plaques confluent; — = no evidence of growth; +++ = slight turbidity with pellicle; N.T. = not tested.

of susceptible staphylococci. After an incubation period of eighteen hours the plates were observed for evidence of plaques. The results are indicated in table 2.

The results of many experiments carried out in the manner described above with four staphylococcus phages lead us to believe that a relationship does exist between actual numbers of bacterial cells of strain C3 and its capacity for absorbing staphylococcus phages. These results also compare favorably with those summarized in table 1. The supernatant of a twenty-four-hour broth culture has no phage-binding power, under the condi-

tions described. However, since the supernatant is not bacteria-free a marked absorption of the phage added to it occurs after appropriate incubating intervals, when growth is observed macroscopically.

When *undiluted* filtrates of mixtures of phage and culture C3 were tested on susceptible strains of staphylococci (dilution being resorted to for accuracy in counting) complete absorption of phage was never observed, there remaining approximately 3 to 5 per cent of the original number of phage corpuscles. Andrewes (1934) in a report dealing with the action of anti-phagic serum stated that it was possible to absorb only 95 per cent of the total number of phage corpuscles. We have been able to corroborate this interesting finding. Methods involving only the use of broth cultures would fail to show that any phage remained unneutralized, and it is specifically by the use of a method involving plaque

TABLE 2

PHAGE M13 IN CONTACT WITH STRAIN C3 FOR TWO MINUTES	PLAQUE COUNT
Resuspended 24-hour culture of strain C3 plus 0.25 cc. of Bp. M13	0
Supernatant of above suspension plus 0.25 cc. of Bp. M13..	197
Savita broth plus 0.25 cc. of Bp. M13 . . . . .	181

demonstration that such unneutralized corpuscles may be detected. It appears that the absorption by strain C3 is comparable to that reported for neutralization by anti-phagic serum.

Filtrates of cultures C3 incubated from periods varying from 24 hours to 27 days lack the ability to diminish staphylococcus phage counts. Unfiltered 20-day-old autolysates also fail to reduce the titer of staphylococcus phages. Spore suspensions have no apparent effect on these same phages, but with the germination of the spores and the subsequent increase in cell numbers one observes the diminution in plaque count.

The ability of strain C3 to absorb staphylococcus phages is not lost when this culture is subjected to autoclaving at 15 pounds for 15 minutes. An agar slant culture was suspended in 5 cc. of saline, and this suspension was autoclaved. A series of tubes containing equal amounts of phage and progressively increasing



amounts of autoclaved bacterial cells were set up and incubated for 20 hours at 35°C. A dilution of  $10^{-5}$  of each mixture *without* filtering was overlayed on a susceptible culture, and after incubation the plate was read for evidence and numbers of plaques. Table 3 gives the results obtained.

One may show absorption of staphylococcus phage by the use of dead bacterial cells providing attention is paid to the number that are utilized. The principle in the cells responsible for this absorption is heat stable. In a study dealing with the absorption of staphylococcus bacteriophages by heat-killed susceptible strains of staphylococci and extracts derived from these strains Rakieten *et al.* (1936) also demonstrated that the absorbing qualities of such cultures and extracts were not impaired on autoclaving at 15 pounds for 15 minutes.

Strain C3 and twenty-one other strains belonging to the *subtilis* group have been tested for their ability to absorb several races of coli, dysentery, proteus and streptococcus phages. No evidence of reduction in corpuscular count was ever obtained, even though some of these phages remained in contact with these cultures for 72 hours. Furthermore, susceptible cultures of *Escherichia coli* and *Salmonella dysenteriae* placed in broth containing proliferating cells of any of these twenty-one strains of *Bacillus subtilis* are completely lysed by their respective phages, the resulting growth being a pure culture of Gram-positive rods. One can not however, prevent the absorption of staphylococcus phages by culture C3 by adding to this mixture an excess of coli, dysentery or streptococcus phages.

All attempts to release and demonstrate staphylococcus phage following absorption by strain C3 have failed. The following methods have been employed to show any evidence of phage that might be released.

1. *Use of an indicator strain.* This consists in touching, as is done in the cross test (Asheshov, 1933), several strains of susceptible staphylococci streaked on an agar plate, with a tiny loopful (loop 1 mm. diameter) of a mixture containing culture C3 and absorbed phage. This method which does not employ filtration, when positive, and small quantities of phage are present, is indi-

cated by a clear zone of lysis on the susceptible strains around the growth of the superimposed contaminating culture. This method is often utilized to determine whether colonies of secondary cultures are free of bacteriophage. No evidence of bacteriophage was obtained when cultures of C3 which had absorbed staphylococcus phages were tested by this method.

2. *The addition of a susceptible culture of staphylococci to a culture of C3 following absorption of a bacteriophage.* All attempts to show that the added culture of staphylococci had been in contact with phage and therefore resistant, or partially so, were negative.

3. A culture of strain C3 which had absorbed bacteriophage was spun in the centrifuge at 3000 r.p.m. for three hours; the

TABLE 3

*Absorption of bacteriophage M13 by autoclaved culture of strain C3*

AMOUNT OF AUTOCLAVE CULTURE ADDED TO 5 CC. OF BROTH	AMOUNT OF Bp. M13 ADDED	PLAQUE COUNT—SUSCEPTIBLE CULTURE Pk
cc.	cc.	
0 1	0 25	194
0 2	0 25	196
0 5	0 25	182
0 7	0 25	161
1 0	0 25	31
2 0	0 25	0
0	0 25	Over 500

supernatant and the sediment were then tested for any trace of phage by a plaque method. No positive evidence of any disunion of phage was ever obtained.

4. *Attempts to produce anti-staphylococcus bacteriophagic serum by injecting rabbits with cultures of strain C3 and absorbed phage.* It was thought that if, in such a mixture, any bacteriophage might be liberated *in-vivo*, an antibody response to such release phage might be obtained. No significant increase over the naturally occurring anti-staphylococcus bacteriophagic principle in the serums (average 1:60) was obtained by this method.

5. *Subjecting to lysis a culture of strain C3 which had absorbed staphylococcus bacteriophage.* An extremely active and specific bacteriophage for strain C3 was isolated from sewage in 1934.

An entire broth culture of this organism which had absorbed phage was lysed, and the filtrate, lysate, or both were tested against several susceptible cultures of staphylococci. All attempts by any method at our disposal failed to reveal that any staphylococcus phage had been obtained from the dissolved culture. On two separate occasions it appeared that indications of phage incompatibility were discovered. In tubes in which equal quantities of staphylococcus and subtilis bacteriophages were placed we observed a complete loss of the staphylococcus phage, whereas the controls containing only the same quantities of each phage were unchanged. Asheshov (1933) has also described instances of phage incompatibility.

During the past three years 27 strains of Gram-positive spore-forming bacilli (aerobic), belonging to the subtilis group have been isolated from agar plate contaminations. Only one of these contaminants, a strain of *Bacillus mycoides* has in any way been able to absorb staphylococcus phages. We have also studied 31 strains of organisms belonging to the subtilis group, which were obtained from Dr. L. F. Rettger's department at the Yale University School of Medicine. From this group, three cultures of *B. subtilis* and one strain of *Bacillus cereus*, partially diminish the plaque count of staphylococcus phages, and two strains, nos. 8, and 79 (*B. subtilis*) are as capable of absorbing staphylococcus phages as strain C3. The remaining 25 strains lack any demonstrable staphylococcus phage-absorbing power. Altogether 58 strains, noted above, and four others including *Pseudomonas aeruginosa*, and *Bacillus pseudotetanicus* have been tested for any capacity to inactivate coli, dysentery, proteus and streptococcus phages. No diminution in plaque count of these phages was ever observed.

It appeared therefore, that we were dealing with another example of so-called non-specific bacteriophagic (staphylococcus) absorption. It is well known that staphylococcus phages are, or appear to be, rendered inactive by a variety of non-specific substances. Shortly after the appearance of the reports by Levine and Frisch (1933 34) and Burnet (1934) dealing with phage inactivation by extracts of susceptible cultures of bacteria,

we prepared extracts of culture C3 and two other staphylococcus phage-absorbing strains of *B. subtilis* by the method of Burnet, in order to determine whether such extracts would act towards phage in the same manner as those prepared from strains of susceptible staphylococci (Rakieten, *et al.*, 1936). Extracts prepared in this fashion lack any phage-inactivating power, either towards staphylococcus phages or toward phage for strain C3.

Our experience with phage has led to the view that only rarely are so-called non-specific factors responsible for phage absorption. In his early studies with phage d'Herelle maintained that there was a definite relation between susceptible bacterial protoplasm and phage absorption. The recent studies with bacterial extracts of susceptible cultures, noted above confirm this viewpoint. It seemed logical therefore to assume that subtilis cultures that have the ability to absorb staphylococcus phages have in their antigenic constitution a factor similar to that of susceptible staphylococci. The demonstration of such a principle, by serological means might serve to explain the reason for phage absorption. This antigenic component, acting in much the same manner as susceptible staphylococcus protein with regard to phage absorption, ought to be detectable in properly prepared anti-serum. Antigenic relationships of widely removed species of bacteria have been demonstrated by Lancefield (1925), Julianelle (1926), and Sugg and Neill (1929).

Young white rabbits were immunized with living cultures of subtilis strains C3 and no. 8 (three with C3; two with no. 8). Prior to the initial injection, these five rabbits were bled from the heart and their serums tested for the presence of natural occurring agglutinins for eight strains of *B. subtilis*, in which were included strains C3 and no. 8. No agglutinins were detected in titers from 1:10 to 1:320. However, all five serums had some naturally occurring agglutinins for our susceptible strains of staphylococci in dilutions ranging from 1:10 to 1:40. After a six weeks course of immunization the animals were bled and agglutinations were done on the homologous strains as well as on several other strains of *B. subtilis* and staphylococci. The results are recorded in table 4.

Both antisera agglutinate homologous antigen to a fairly high titer. Anti-C3 serum in addition also agglutinates several other members belonging to the subtilis group (*B. subtilis* Marburg, no. 81, air no. 1, and *B. mycoides* Rhgld). However, while these strains appear to have agglutinins similar to strain C3, as demonstrable above, the very active phage against strain C3 has

TABLE 4

STRAIN	1 50	1 100	1 200	1 400	1 800	1.1600	1.32000
Agglutinative power of rabbit serum (pooled) anti- <i>B. subtilis</i> 8							
<i>B. subtilis</i> No. 8....	4	4	4	4	4	4	2
<i>B. subtilis</i> C3.....	4	4	4	2	1	0	0
<i>B. subtilis</i> Li6.....	0	0	0	0	0	0	0
<i>Staph. alb.</i> V.....	4	4	4	4	1	0	0
<i>Staph. aur.</i> Vnt.....	0	0	0	0	0	0	0
Agglutinative power of rabbit serum (pooled) anti- <i>B. subtilis</i> C3							
<i>B. subtilis</i> C3 .....	4	4	4	4	4	4	4
<i>B. subtilis</i> 81 .....	4	4	4	4	4	4	0
<i>B. subtilis</i> 8.....	0	0	0	0	0	0	0
<i>B. subtilis</i> air No. 1.....	4	4	3	0	0	0	0
<i>B. subtilis</i> Marburg.....	4	2	1	0	0	0	0
<i>B. subtilis</i> Jordan.....	0	0	0	0	0	0	0
<i>B. subtilis</i> Novy.....	0	0	0	0	0	0	0
<i>B. subtilis</i> N. Y. No. 82. ....	0	0	0	0	0	0	0
<i>B. subtilis</i> C9.....	0	0	0	0	0	0	0
<i>B. mycoides</i> Rhgld .....	2	2	3	0	0	0	0
<i>Staph. alb.</i> V.....	4	4	4	3	1	0	0
<i>Staph. aur.</i> Grazl.....	2	1	0	0	0	0	0
<i>Staph. alb.</i> Grazl.....	4	4	4	0	0	0	0
<i>Staph. aur.</i> Vnt.....	4	3	3	1	0	0	0
<i>Staph. Gec.</i> (resistant).....	0	0	0	0	0	0	0
<i>Staph. Grnspar</i> (resistant .....	0	0	0	0	0	0	0

4 = complete agglutination; 0 no observable agglutination.

no action whatsoever on these same strains. Nor is the anti-C3 phage absorbed to any degree when placed in a medium in which any of these serologically related strains are proliferating. The serum containing anti-no. 8 antibody is able, to a fair degree, to agglutinate subtilis C3, but a reciprocal action is not demonstrable by the anti-C3 serum toward subtilis no. 8.

Both anti-subtilis serums also possess agglutinins increased to a significant degree over those found naturally for phage-susceptible strains of staphylococci. These staphylococcus cultures, as well as extracts prepared from them, will absorb the same staphylococcus phages that are absorbed by subtilis cultures C3 and no. 8. Neither of the two phage-resistant strains of staphylococci are agglutinated by these anti-subtilis serums. While it may be assumed that during the course of immunization with subtilis cultures the naturally occurring staphylococcus agglutinins are also stimulated, this criticism is not entirely warranted because of the fact that the phage-resistant strains of staphylococci are not affected; especially since sera prepared against some of

TABLE 5

	SERUM DILUTIONS			
	1 100	1 200	1 400	1 800
(a) Anti-subtilis serum (C3, diluted 1:50) absorbed with <i>Staph. Vnt.</i>				
Absorbed serum with <i>Staph. Vnt</i> . . . . .	0	0	0	0
Absorbed serum with <i>B. subtilis</i> C3... . .	4	4	4	4
(b) Anti-subtilis serum (C3, diluted 1:50) absorbed with <i>B. subtilis</i> C3				
Absorbed serum with <i>Staph. Vnt</i> . . . . .	0	0	0	0
Absorbed serum with <i>B. subtilis</i> C3....	0	0	0	0

4 = complete agglutination; 0 = no agglutination.

our phage-susceptible strains of staphylococci do agglutinate these resistant strains. Also, sera prepared against the resistant strains will also agglutinate phage-susceptible cultures. In this instance agglutinogenic similarity is not entirely associated with phage susceptibility as Burnet (1933) points out. On the basis of the above results there appears to be some justification for concluding that there is a correlation between the staphylococcus phage-binding power of certain members of the subtilis group of bacilli and the ability of these same strains to produce agglutinins for phage-susceptible strains of staphylococci. It appears that in the antigenic components of these strains there is a staphylococcus-like stimulant, and that this heterogenetic antigen be-

haves under certain conditions like staphylococcus substance so that it may be detected by the absorption of staphylococcus phages.

That the staphylococcus agglutinins in the serums are the minor ones may be proven by an agglutinin absorption method. The results set forth in table 5 indicate what takes place when an anti-subtilis serum is absorbed with *Staphylococcus aureus* Vnt and *B. subtilis* C3.

A relationship between strain C3 and susceptible cultures of staphylococci may also be demonstrated by placing the phage active against this strain of *B. subtilis* in a medium containing an actively growing culture of susceptible staphylococci. Under these conditions a considerable amount of this subtilis phage is absorbed in much the same manner as the staphylococcus phages are absorbed by an actively growing culture of strain C3. No evidence of subtilis phage absorption has as yet been obtained when lyso-resistant strains of staphylococci were utilized.

#### DISCUSSION

In general, bacteriophages regardless of type tend to become absorbed by susceptible protoplasm (bacterial), either living, heat-killed, or in the form of a watery extract. Staphylococcus phages, in particular, while they also conform to the general principles stated above, in addition may also be absorbed by such cellular elements as yeast and white blood cells (d'Herelle and Rakieta, 1935). That white blood cells may also take up other viruses has been recorded by Douglass and Smith, (1930), Daubney (1928) and Schein (1917). Coli bacteriophages as well as other phages acting especially on the enteric group of bacteria are scarcely affected when allowed to remain in contact with leucocytes. Lysis of susceptible *E. coli* readily occurs by bacteriophages in concentrations of pus that have a definite inhibitory effect on staphylococcus phages.

Bacteriophages attacking staphylococci are also absorbed and inactivated irreversibly by certain bacteria not ordinarily regarded as related to staphylococci. These organisms, described in the present report, are members of the subtilis group. It is

believed that some portion of their antigenic constitution resembles that of susceptible staphylococci, and that it is this factor which is responsible for their staphylococcus-phage-absorbing quality. This factor also resembles the factor responsible for phage absorption by susceptible staphylococci in its heat stability. Finally this factor is able to stimulate formation of an antibody that reacts with susceptible staphylococci, and may be identified by serology.

It is becoming more and more evident that bacteriophages are useful agents in the detection of antigenic differences in closely allied strains of bacteria (Levine and Frisch, 1934, Burnet, 1934, Craigie, 1936, Almon and Stovall, 1936, and Sertic, 1936). The present report indicates the usefulness of bacteriophage in detecting antigenic similarities in strains widely removed from each other.

Union between phages and susceptible bacterial protoplasm, irrespective of the state in which it is presented (living, heat-killed, or in the form of an extract) takes place very rapidly at incubator temperature, the largest proportion of phage being taken up in less than thirty minutes. Once the phage has been absorbed it is difficult, under ordinary conditions, to effect any release. In all instances where staphylococcus phages have been absorbed by strains of *B. subtilis* all of the several methods utilized to free absorbed phage failed to show that any lytic agent had become disunited.

Burnet in a series of reports (1929-33) dealing with phages attacking bacteria of the enteric group has shown that a relation exists between the heat-stable agglutinogenic constitution of these bacteria and their susceptibility to phage. With regard to the phage isolated for *B. subtilis* C3 this relationship does not hold, since organisms that have agglutinogens in common with strain C3 are not attacked by this phage, nor is this phage absorbed by placing it in contact with any of these related cultures. However, it is not our purpose to conclude on the basis of the results with one phage that a relation in the subtilis group does not exist that is comparable to that in the *Salmonella* group as described by Burnet.



Our failure to demonstrate that extracts of culture C3 are able to inactivate staphylococcus phages may be due to the fact that the methods used to prepare such extracts were faulty. Extracts so prepared had no phage-inhibiting action toward the homologous subtilis phage.

From a practical standpoint the results described may be of some value in explaining the failure to find staphylococcus phages in sources that are rich in coli and dysentery bacteriophages.

### CONCLUSIONS

1. Staphylococcus bacteriophages are absorbed by living or heat-killed cultures of bacteria belonging to the subtilis group.

2. The bacilli are not influenced by their contact with staphylococcus phages.

3. Absorbed bacteriophage can not be detected, even after lysis of the absorbing strain.

4. Coli, dysentery, proteus, and streptococcus phages are not affected by contact with these same strains.

5. There appears to be an antigenic relationship between strains of *Bacillus subtilis* that are capable of absorbing staphylococcus phages, and phage-susceptible strains of staphylococci.

6. Phage-susceptible strains of staphylococci also absorb a subtilis phage.

7. Bacteriophage may be a very valuable aid in studying the antigenic composition of bacteria.

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# ANTIBIOSIS IN THE COLON-TYPHOID GROUP

## I. GROWTH CURVES OF TWO STRAINS IN A SYNTHETIC MEDIUM<sup>1</sup>

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Associations of typhoid or paratyphoid bacilli with *Escherichia coli* have been studied by many workers for the light they might throw on the pathogenesis of enteric fevers and for their connection with the laboratory problem of isolating the intestinal pathogens from feces. The general history of our knowledge of bacterial associations has been reviewed by Seitz (1927) and by Holman (1928). There will be cited here only a few papers dealing with associations of the species mentioned.

An early study was that of Chatterjee (1909), who noted that typhoid and paratyphoid bacteria failed to multiply when inoculated into media previously seeded with colon bacilli.

Space does not permit more than the mention of the work of van der Reis (1923), Mazzeo (1926), Broom (1929), Vignati (1926) (1928), Dimitrijevic-Speth (1930), Schilling and Califano (1930), and Peretz and Slawsky (1933).

Nissle (1916) introduced a small inoculum of colon bacilli into a rapidly growing broth culture of typhoid bacilli, incubated overnight, and streaked out the mixture on Endo plates. The colonies of each species were counted in suitable areas on the plates and the result expressed as the number of typhoid per 100 colonies of colon bacilli. This ratio Nissle termed the "antagonistic index" of the association. Prell (1919), Worpenberg

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(1924), Pfalz (1926), Tey (1927), Hashimoto (1927) and Koch and Kramer (1931) are among the many who have followed Nissle's method, although often introducing modifications.

Wikullil (1932) (1934) considered the problem in the light of Bail's (1929) work on the factors controlling the size of bacterial populations. Bail had pointed out that the growth curves of many species of the colon-typhoid and other groups exhibited a characteristic maximum cell concentration point. Wikullil suggested that this characteristic maximum number might in some manner determine the behavior of the strains in an association.

A somewhat different approach was made by Etinger-Tulczynska (1932) (1934), and Neufeld and Kuhn (1934). These workers considered that the controlling factor in antibiosis was the ratio between the two species which was established at the time of inoculation. This they termed the "minimum inhibitory ratio." If two strains were seeded into a medium in proportions which equalled or exceeded this ratio, the more numerous strain always rapidly suppressed the other.

Many theories have been offered to account for the phenomenon of antibiosis. One of the oldest was the theory that more or less specific "excretory products" were produced by the stronger organism in order to destroy the weaker. Hashimoto was inclined to favor this explanation. Broom believed that exhaustion of essential food materials by the stronger strain was the cause of antibiosis. Mazzeo and several others attributed inhibition to unfavorable changes in the pH of the medium. The whole question of the "isoantagonism and heteroantagonism" of culture filtrates is closely related to the problem of what mechanism underlies antibiotic action, but for the sake of brevity this aspect of the subject is not reviewed here.

Worpenberg held that antibiosis depended on the relative vigor of growth of the associants. Pfalz attributed antibiosis to differences in the metabolic processes of the associated strains. Wikullil held that the mechanical factors postulated by Bail for pure cultures caused inhibition in mixed cultures. Neufeld and Kuhn suggested that there was some form of interaction between the

dividing cells which led to a "direct antagonism" in which metabolic products and nutritional requirements played no part.

This brief summary indicates the unsatisfactory state of our knowledge of antibiosis in the colon-typhoid group. Various methods have been employed in establishing and analyzing associations and even the use of similar methods by different workers has not resulted in similar conclusions. Experiments to be presented in a subsequent paper indicated that the methods of Nissle, Wikullil, and Etinger-Tulczynska did not individually give an accurate and complete picture of the population changes in the association under study. A more complete picture is given by the growth curves of an association and of the pure cultures of the species used in the association. The growth of each species in an association can then be compared with its growth in a pure-culture control. This paper presents the results of a study of one association by this method.

#### METHODS

A synthetic medium was adopted in order to have the food supply under control. Braun and Goldschmidt's (1929) formula was modified as follows:

Aspartic acid . . . . .	1 0 per cent by weight
m/5 $\text{KH}_2\text{PO}_4$ solution . . . . .	2 0 per cent by volume
m/5 $\text{MgSO}_4$ solution . . . . .	0 1 per cent by volume
NaCl . . . . .	0 5 per cent by weight
Brom thymol blue, 1.6 per cent alcoholic	0 1 per cent by volume

The aspartic acid and NaCl were dissolved in about half the required amount of water. The other substances were added from stock solutions. The pH was adjusted to 7.0 with N/1 NaOH. The solution was made up to volume, tubed in 5-ml. quantities, and autoclaved 10 minutes at 15 pounds.

Aspartic acid is the sole source of both carbon and nitrogen in this medium. Under such restricted conditions of nutrition, differences in the availability of the food supply might determine the relationships of organisms in association. It is therefore of importance that both the species used in the experiments

were able to subsist well during serial transfers in this simple medium.

A number of strains of *Escherichia coli* and *Salmonella schottmuelleri* were grown in the medium for varying periods of time. One strain of each of these species was selected for the experiments and they were carried in the medium for 15 months during which 149 transfers were made. There was no evidence of a period of acclimatization to the medium, nor any sign of degeneration due to absence of essential nutrients. The maximum live cell concentrations of the two strains were about the same as in nutrient broth, although the rate of growth was slower and death more rapid. Increasing the amount of aspartic acid to four times the specified concentration did not materially alter the growth curves. The cultures dissociated to SR and R colony types resembling those described by Torrey and Montu (1936), but no changes in biochemical properties were observed and the tendency to dissociation did not seem greater than that of the same strains in nutrient broth. Complete dissociation was prevented by plating frequently and selecting S-type colonies.

When the initial number of organisms was small, growth in the synthetic medium was frequently slow in beginning. This effect was studied carefully because a prolonged lag phase of this type might be mistaken for inhibition in antibiosis experiments. There were definite indications that the prolonged lag was the result of an unfavorable oxidation-reduction potential of the medium, since it could be eliminated either by the addition of small amounts of cysteine or by larger inoculations. The latter method was adopted.

In order to minimize errors arising from variations in counts in duplicate tubes, the counts were made on the pooled contents of 3 to 5 tubes. Dilutions were prepared in weakly buffered distilled water which had been sterilized in bulk and distributed aseptically in the required amounts just before use. Plates were inoculated by flooding as described by Anderson and Stuart (1935). The eosin methylene-blue agar used for plating had the following formula:

Beef extract.....	3 0 grams
Peptone.....	10 0 grams
Lactose.....	10 0 grams
Agar.....	15 0 grams
Eosin 5 per cent aq.....	3 5 ml.
Meth. blue 1.25 per cent aq.....	3 2 ml.
Aq. dist.....	1 0 liter

The reaction was not adjusted. The medium was dispensed in amounts of approximately 150 ml. in bottles and autoclaved for 12 minutes at 15 pounds.

The growth curves were followed for 72 hours by means of periodic plate counts. A sufficient number of tubes were inoculated with 1 to 3 drops of 24-hour growths of the individual cultures, or of both in the case of the mixed cultures, and incubated at 37°C. To make a count, several tubes of a culture were pooled and serial dilutions prepared. Four plates were flooded with 1 ml. each of a dilution which would yield 50 to 150 colonies per plate. After 24 hours incubation the colorless *S. schottmuelleri* colonies were well differentiated from the black *E. coli* colonies, and a differential count was easily made.

#### RESULTS

The arithmetic means of the counts secured in 16 experiments are tabulated in table 1 and graphed in figures 1 to 3.

The only variation in the conditions of these experiments was in the relative numbers of the two species in the inoculum. Small differences in this respect had no evident effect on the history of the association. In certain experiments a large enough number of tubes of the association were inoculated so that the growth curves of the association could be determined simultaneously in triplicate.

The mean growth curves of the pure cultures (fig. 1) present certain interesting features. *S. schottmuelleri*, although it grew somewhat more slowly, actually reached a higher maximum cell concentration than *E. coli*. The curves for both species rose and declined steeply, making the limit of multiplication a point (Bail, 1929) rather than a phase of significant duration (maximum stationary phase of Buchanan).



In the association (fig. 2) the maximum numbers of both species were reduced, with *S. schottmuelleri* apparently undergoing the greater inhibition. There was a definite period of depression at 24 to 36 hours in the curve for *S. schottmuelleri*, coinciding with the period during which the *E. coli* count was near its maximum.

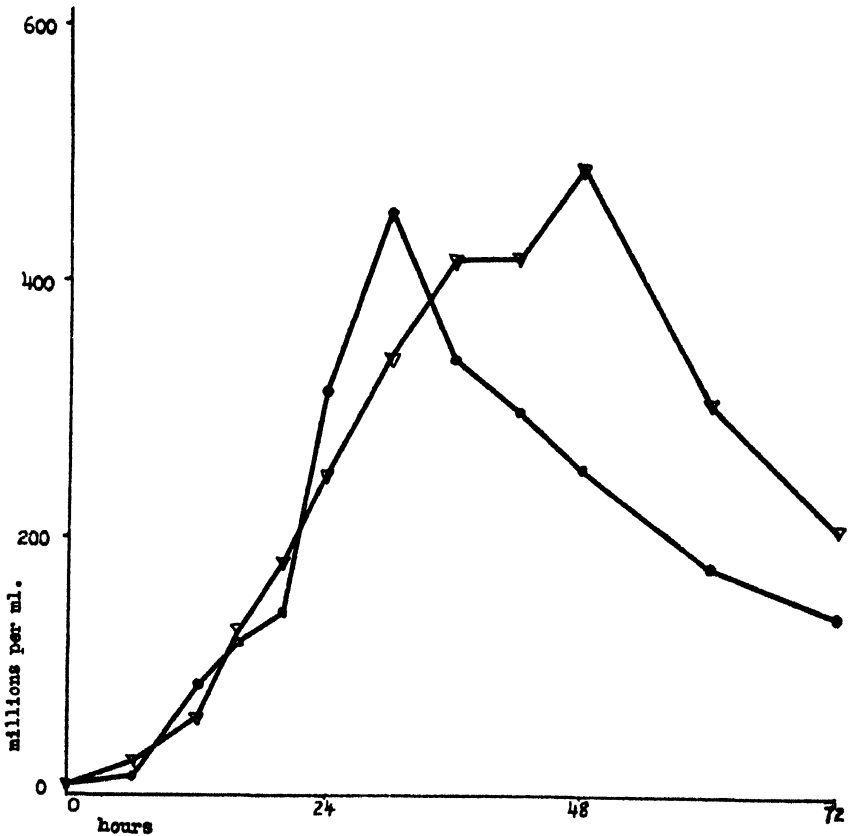


FIG. 1. MEAN GROWTH CURVES OF PURE CULTURES OF *E. COLI* (●) AND *S. SCHOTTMUELLERI* (▽)

The depression was transitory and the *S. schottmuelleri* count increased again when the *E. coli* count decreased. This period of depression occurred regularly, unless the initial number of *S. schottmuelleri* was small, in which case there was usually little evidence of multiplication of that species until the *E. coli* count

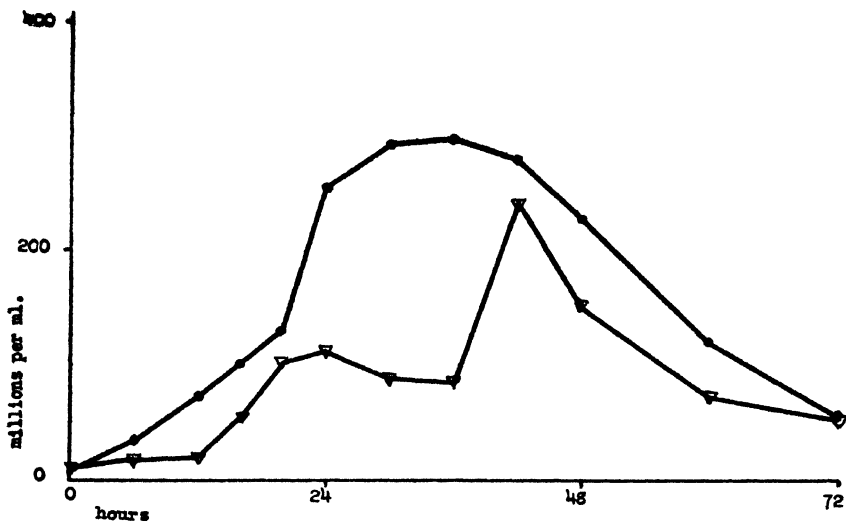


FIG. 2. MEAN GROWTH CURVES OF THE ASSOCIATION *E. COLI* (●)-*S. SCHOTTMUELLERI* (▽)

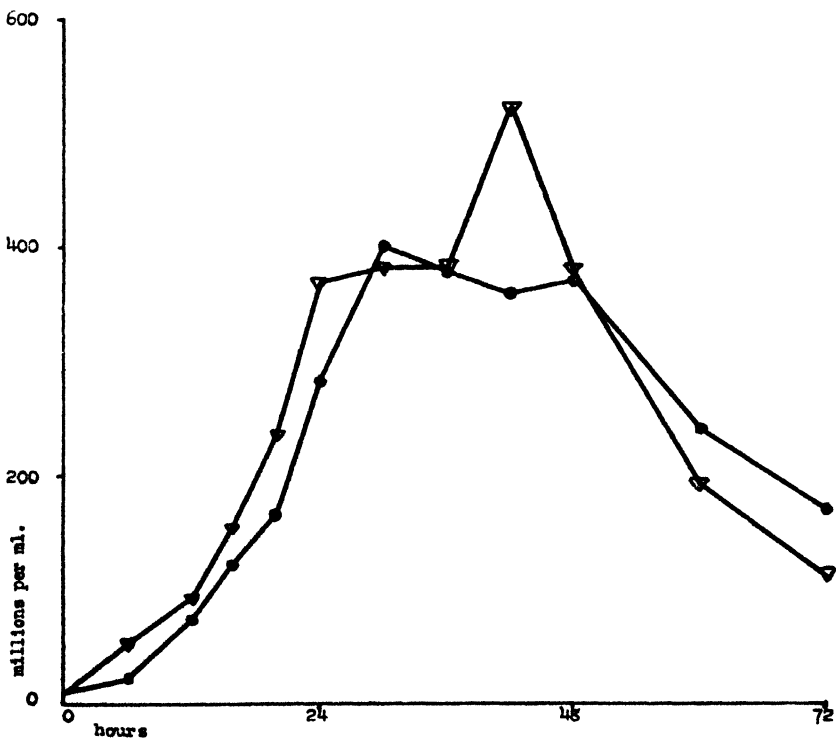


FIG. 3. MEAN TOTAL NUMBERS OF BACTERIA IN PURE CULTURE (●) AND ASSOCIATION (▽)

had passed its maximum point. Depression occurred even in experiments in which *S. schottmuelleri* was originally present in greater numbers than *E. coli*. It might be expected that this initial preponderance would enable *S. schottmuelleri* to inhibit *E. coli*. On the contrary, the depression of *S. schottmuelleri* developed as usual and *E. coli* soon predominated.

In figure 3 are presented the curves of total live cell concentrations. These were secured for the association by adding together the *E. coli* and *S. schottmuelleri* counts. The total counts in pure cultures were calculated as one half the sum of the counts

TABLE 1

Mean counts and totals of pure and mixed cultures of (A) *Escherichia coli* and (B) *Salmonella schottmuelleri* in millions per milliliter

AGE	PURE CULTURES		ASSOCIATION		TOTAL COUNTS	
	A	B	A	B	Pure	Mixed
hours						
0	7 3	8 5	7.3	8 5	7 9	15 8
6	15 6	26 4	35 0	18 5	21 0	53 5
12	85 2	61 3	73 0	19 8	73 3	92 8
16	120 0	126.2	100 4	55 0	123 1	155 4
20	143 5	183 8	131 4	103 4	168 7	234 8
24	315 5	250 4	255 3	113 1	282 9	368 4
30	459 0	343 1	293 6	89 6	401 1	383 2
36	340 0	418 7	297 1	85 7	379 4	383 8
42	300 0	419 5	280 4	243 5	359 8	523 9
48	256 7	489 6	228 6	149 6	373 2	378 2
60	178 5	305 1	119 8	72 2	241 8	192 0
72	136 0	207 6	61 1	52 4	171 8	113 5

of the two pure cultures, since this sum represented the number of bacteria in 2 ml. of medium. The highest point on the curve of the pure culture totals was determined by the *E. coli* peak at 30 hours. There was a second but lower peak at 48 hours, corresponding to the maximum cell concentration in the *S. schottmuelleri* culture. The peak of the curve of total numbers in the association came at 42 hours, the maximum cell concentration point of *S. schottmuelleri*. It was preceded by a lower zone of nearly constant numbers which was the resultant of slowly increasing *E. coli*

and slowly decreasing *S. schottmuelleri* counts. In general, the curves of total numbers resemble one another, the chief differences being determined by the time variations in location of the maximum cell concentrations.

As the results in the 16 individual experiments varied, the significance of the mean curves reported was examined by statistical methods. The coefficient of variability of each count recorded in table 1 was determined by the usual formula:

$$C.V. = \frac{100 \Sigma(d^2)}{M}$$

TABLE 2

Counts of associations of (A) *Escherichia coli* and (B) *Salmonella schottmuelleri* in millions per milliliter in an experiment carried out in triplicate

AGE	NO. 1		NO. 2		NO. 3	
	A	B	A	B	A	B
hours						
0	9 0	13 0	9 0	13 0	9 0	13 0
2	17 3	9 3	18 3	9 3	22 0	6 8
12	73 0	10 0	100 0	3 0	85 0	12 5
24	203 0	10 0	373 0	13 0	373 0	15 0
30	372 5	10 0	400 0	10 0	403 0	15 0
36	412 5	27 5	363 0	33 0	367 0	37 0
48	357. 5	137 5	317. 5	103 0	287 5	52 5
60	263 0	137 0	287 0	200 0	170 0	47 0
72	176 0	157 0	151 7	145 0	*	*
84	155 7	234 0	100 0	167. 0	81 3	95 0
96	51 3	350 0	30 3	300 0	22 0	250 0

\* Lost.

in which the sum of the squares of deviations from the mean is divided by the mean itself and the quotient multiplied by 100 in order to express the results in per cent. The mean coefficient of variability was  $\pm 65$  per cent.

There were rather large variations in the amounts of growth of *S. schottmuelleri* during the period in which the number of *E. coli* was declining. At these times *S. schottmuelleri* was growing in a medium partially although not completely depleted of food, and altered by *E. coli* metabolites, especially alkali which was concen-

trated enough to cause a pH of 8.4 to 8.8, a value close to the alkaline limit of tolerance of *S. schottmuelleri*. However, variations in the amount of growth of the latter during this period were observed even in duplicate experiments. As an example, data are presented in table 2 for the mixed cultures of one experiment in which three association growth curves were determined simultaneously. In general, these curves conform to the mean curves of table 1, but they indicate the irregularities encountered in the experiments from which the averages in table 1 were calculated. Individually, these variations are of little significance when the number of observations is sufficient, but a chance encounter with an extreme case might in the absence of further data lead to erroneous conclusions as to the relationships between two associated organisms.

Uncompleted studies employing other strains of *E. coli* and *S. schottmuelleri* suggest that similar results can be secured when the same technic is used with other strains. No strains were encountered which were of the strongly antibiotic type of colon bacilli named "Mutaflor" by Nissle, and none of the *S. schottmuelleri* strains studied were able to inhibit the growth of *E. coli* under the conditions of these experiments.

#### DISCUSSION

A study of the literature would lead to the expectation that the association of *E. coli* and *S. schottmuelleri* would exhibit bactericidal action by the first with annihilation of the second organism as its result. This was not observed. The depression of *S. schottmuelleri* during the period in which the *E. coli* count is near the maximum cell concentration resembles the anticipated bactericidal effect. The depression is, however, only temporary and *S. schottmuelleri* is not exterminated. If any bactericidal substance is present it must be dilute and readily inactivated.

The tendency shown in the growth curves is toward a succession of the species rather than outright competition for existence. This has not been recognized generally as a form of antibiosis, although workers (e.g., Topley and Fielden, 1922) have long realized that certain organisms which can not be isolated from a

young mixed culture may become the predominant type when the association is older. Certain of the 16 experiments showed the succession of the species more distinctly than appears in the average data in table 1. In those cases, the *S. schottmuelleri* count generally remained so low during the growth of *E. coli* that plates of  $10^{-7}$  dilutions of the mixed culture usually showed no colonies of *S. schottmuelleri*, or at most one or two. In the absence of further evidence these results would certainly be interpreted as indicating the practical annihilation of *S. schottmuelleri*. It is very important, therefore, that a secondary growth of *S. schottmuelleri* always developed in these associations, proving that the annihilation was apparent rather than real.

At present antibiotic activity cannot be expressed quantitatively with any accuracy. The numerical ratio between two associated species (antagonistic index) depends largely on the age of the culture observed, as can be seen from the figures presented. It is therefore misleading to state on the basis of a single observation that one species is antagonistic to another. The evidence does not suggest that in the association studied antibiosis is determined by the initial numbers of each species, as some workers have contended. Nor is there simultaneous growth and mutual inhibition proportional to the maximum cell concentrations of the strains, as described by Wikullil.

In an attempt to clarify the relationships between the two associants the areas under the growth curves were compared. The area was estimated by determining the average count for a time interval and multiplying this by the length of the interval in hours. The product expresses numerically the existence of the bacterial cells in the specified length of time. It has been termed "cell hours" by Carpenter, Fulton and Stuart (1935). It indicates total existence rather than rate of increase or relative numbers of organisms, and is therefore distinct from the count itself and from the growth rate of the culture. When the cell-hour values for all the time intervals in an experiment are added together the sum is the total cell hours for the growth cycle. It expresses concisely the extent to which a strain of organisms has existed in a culture during the period of observation.

Calculation of the total cell hours for the growth curves recorded in table 1 bears out the evidence of inspection of the curves themselves. In pure cultures *S. schottmuelleri* grows better than *E. coli*. The total cell hours for *E. coli* in pure culture is in round numbers  $15,700 \times 10^6$ , while for *S. schottmuelleri* it is  $18,800 \times 10^6$ . In the mixed cultures the corresponding values are: *E. coli*  $11,800 \times 10^6$ ; *S. schottmuelleri*  $6,620 \times 10^6$ ; total  $18,420 \times 10^6$ .

Several comparisons are possible in analyzing these values. (1) The ratio of the total cell hours of one species in mixed culture to the total cell hours of the same species in pure culture will indicate one form of inhibition. This ratio is for *E. coli* 0.74, for *S. schottmuelleri* 0.35. In other words, both organisms grew less in the association, *E. coli* averaging about three-fourths as much growth as in pure culture, and *S. schottmuelleri* about one-third. (2) The relative growth of the cultures as wholes can be expressed by the ratio of the total cell hours in mixed culture to the total cell hours in pure culture. This ratio is 0.535, and indicates that growth of the two species in the association is roughly half as great as their growths separately in pure cultures. (3) It was pointed out in connection with figure 3 that the sum of the pure-culture counts refers to bacteria per 2 ml. of medium. The total cell hours for pure cultures as previously calculated also refers to twice as much medium as the total cell hours for the association. If this difference is corrected by dividing the total cell hours for pure culture by 2, the ratio between total cell hours of the pure and mixed cultures becomes 1.07. (4) The fraction of cell hours contributed to the totals of the mixed cultures by one of the associates, compared to the fraction contributed by the same species to the total cell hours of the pure cultures, indicates antibiosis in another way. Since inhibition of *S. schottmuelleri* is the question at issue, the fractions of the total cell hours contributed by that species were calculated. The fraction is, for pure cultures 0.545, for the association 0.36. In other words, over half of the total cell hours of both pure cultures represents *S. schottmuelleri* hours. In mixed culture the growth of *S. schottmuelleri* accounted for only slightly more than one third of the total cell hours.

These calculations show that the growth in association by both

species, as measured in cell hours, was slightly more than half (53.5 per cent) of the growth in the two pure cultures. Eliminating the difference in volume of the environment in pure cultures and the association, growth in the association was 7 per cent greater than in pure cultures. The difference is presumably negligible. The figures also show that while both species were inhibited in the association, *S. schottmuelleri* was relatively more inhibited than *E. coli* (65 per cent as compared with 26 per cent inhibition of *E. coli*). The fourth calculation shows that *S. schottmuelleri* in mixed culture was inhibited to such an extent that it produced 18.5 per cent less cell hours than would be predicted on the basis of its behavior in pure culture.

This figure for the inhibition of *S. schottmuelleri* is not as large as might be assigned after simple inspection of the growth curves of the association. It is not as large as would be indicated by determination of the antagonistic index at approximately the time when *E. coli* reaches its maximum cell concentration. The treatment of antibiosis in the case presented here differs from previous methods in comparing the association with pure culture controls and in considering the entire growth cycle rather than an arbitrary point on it. The results secured raise doubt as to the existence of antibiosis in the association studied. Standards of comparison are lacking, but 18.5 per cent inhibition of *S. schottmuelleri* does not appear to indicate a high degree of antibiotic action by *E. coli*.

#### SUMMARY

1. The growth cycle of a selected strain of *Escherichia coli* and one of *Salmonella schottmuelleri* in association in a synthetic medium indicates a tendency for the species to succeed each other in the order named.

2. During the early growth of the association, *S. schottmuelleri* is inhibited to a marked degree and the population consists largely of *E. coli*.

3. After *E. coli* passes its maximum cell concentration, a more or less luxuriant growth of *S. schottmuelleri* develops, and the population in old associations consists largely of *S. schottmuelleri*.



4. Considering the growth cycle as a whole, and in comparison with pure culture controls, there is only slight inhibition of *S. schottmuelleri* by *E. coli* in the association studied.

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# "CHROMIUM-SULPHURIC ACID" METHOD FOR ANAEROBIC CULTURES

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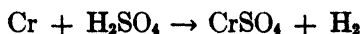
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## PRINCIPLE

When chromium metal powder is added to sulphuric acid, the following reactions take place:

1. Chromium reacts with sulphuric acid, forming chromous sulphate with vigorous evolution of hydrogen:



2. The chromous sulphate in the presence of sulphuric acid absorbs oxygen and is readily oxidized to chromic sulphate:



Thus, chemical reactions involving only two reagents may serve two purposes: the displacement of air by the generated hydrogen, and the absorption of the residual oxygen by the chromous compound.

## APPARATUS

An apparatus for the application of the foregoing principle should have the following features:

1. It should be capable of being hermetically sealed.
2. It should be provided with an outlet for the egress of the generated hydrogen. An arrangement should be made for the closure of this outlet whenever necessary.
3. As the bottom of the apparatus is to be filled with the reagents, a provision must be made for an acid-proof stand, elevated above the surface level of fluid, in order to hold the culture tubes

and dishes. A Novy jar or one of the usual desiccators with ground-glass stopcock and glazed porcelain plate, resting on an inner flange, fulfills these requirements. However, it is relatively simple to construct for the same purpose an improvised apparatus from ordinary Mason fruit-preserving jars which have a threaded metal cap. The jar is hermetically closed by putting the sealing mixture between the jar and cap and covering the junction with several rounds of adhesive tape. A small perforation is made in the center of the cap to serve as an outlet for the gas. When required, this may be sealed with modeling wax (plasticene).

#### REAGENTS

1. Chromium metal powder.
2. Sulphuric acid, 15 per cent aqueous solution by volume.
3. Sealing mixture, consisting of 90 parts of white petrolatum and 10 parts of beeswax, melted together and allowed to re-solidify.
4. The usual methylene blue indicator:  
Solution A: 3 cc. 0.5 per cent aqueous solution of methylene blue, diluted to 100 cc. with water.  
Solution B: 6 per cent aqueous solution of glucose with a crystal of thymol added for preservation.  
Solution C: 6 cc. N/10 NaOH, diluted to 100 cc. with water.  
Immediately before use, a mixture of equal parts of the three solutions is prepared.

#### TECHNIQUE

1. Carry out the first four steps in rapid succession.
  - a. Pour on the bottom of the jar the 15 per cent solution of sulphuric acid (100 cc. per liter capacity of the jar) and add chromium metal powder (5 grams per liter capacity).
  - b. Place the test tubes and the Petri dishes with the cultures on the stand.
  - c. Introduce a tube filled with the methylene blue indicator.
  - d. Place the lid on the jar, taking care to open the gas outlet.
2. After the initial vigorous evolution of hydrogen has subsided

and only a mild effervescence of the fluid on the bottom of the jar is observable, close the gas outlet.

3. Place the sealed jar in the incubator.

4. On opening the jar, after the desired period of incubation, avoid exposure to flame as the jar and culture tubes are saturated with hydrogen.

#### ADVANTAGES OF THE METHOD

1. The method is simple and does away with expensive, complicated equipment. There is no need for an outside generator of hydrogen, for a vacuum pump to exhaust the air, for a device of palladinized asbestos wool, etc.

2. The chromous sulphate reagent absorbs the oxygen at a rate far superior (more than 40 times<sup>1</sup>), to that of the alkaline pyrogallate which, at present, is used for that purpose. Consequently, the jar is rapidly freed from oxygen. The best evidence of this fact is decoloration of the methylene blue indicator which occurs within a few minutes instead of several hours, as is the case with the methods in ordinary use.

3. The suggested amounts of chromium and sulphuric acid are double the amount theoretically required. Therefore, an excess of an active oxygen absorbent remains available during incubation, as a safeguard against possible traces of oxygen which were dissolved in the nutrient media or which could seep into the apparatus. The exclusion of free oxygen, even after prolonged incubation, is indicated by the continued decoloration of the methylene blue.

4. Inasmuch as no vacuum is created during any step of the method, the Petri dishes can be placed in inverted position and the growth of isolated colonies is not impaired by collection of moisture on the surface of the agar.

5. During the incubation, the pressure of hydrogen inside the apparatus remains slightly increased which serves as a protection against penetration of air from the outside.

<sup>1</sup> Stone, H. W. 1936 The use of chromous sulfate in the removal of oxygen from a stream of gas. A comparison with other oxygen absorbents. Jour. Amer. Chem. Soc., 58 (Part 2), 2591-2595.

6. The presence of fluid on the bottom of the jar prevents the dehydration of the media even on prolonged incubation.

7. The method is efficient. Cultures on liquid media and good surface growth on agar can be easily obtained even in the case of strict anaerobes.

#### SUMMARY

An anaerobic method is described, based on the reaction of chromium metal with sulphuric acid. The reagents are placed at the bottom of a culture vessel which can be hermetically sealed, and which is provided with an outlet for gas. The generated hydrogen escaping through the open outlet displaces the air. After the outlet has been closed, the residual oxygen is absorbed by the chromous sulphate formed.

## STUDIES ON ANAEROBIC BACTERIA

### XI. ON THE PROPERTIES OF THE H AGGLUTINOGENS OF A MESOPHILIC AND A THERMOPHILIC SPECIES<sup>1</sup>

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It is one of the bases of the double receptor theory that the H and O agglutinogens differ in resistance to heat. Yet the degree of difference is not clear. It is not certain, for example, whether the H substance of different species of bacteria is of the same lability; or whether the occasional report of survival of H through a heat treatment, which is expected to inactivate it, is explained by some protective condition during heating.

Most records of the thermal relations of H antigens concern the pretreatment of suspensions intended for animal injection. Felix and Robertson (1928) and Condrea (1930) used 1 or 2 hours at 60°C., in treatment of *Clostridium tetani*; Tulloch (1918) for the same purpose 30 minutes at 60°C. Henderson (1932) treated *Clostridium chauvoei* for 1 hour at 60°C. Schoenholz and Meyer (1923, 1925) also chose 1 hour at 60° for *Clostridium botulinum* but Starin and Dack (1923) increased the treatment to 10 to 15 minutes at 70°C. and Falk and Powdermaker (1925) to 30 minutes at 78°C. For non-spore-forming organisms, the treatments have been less drastic. Nelson (1928) with *Salmonella paratyphi* used 1 hour at 56°C. and Abdoosh (1932) for *Vibrio cholerae* only 30 minutes at 58°C. Occasionally when there is no question of pathogenicity of the organism, a heat treatment is given, apparently to prevent autolysis of antigen suspensions. Thus, a treat-

<sup>1</sup> This investigation has been aided by a grant from the Wisconsin Alumni Research Foundation.



ment of 1 hour at 58°C. was given *Bacillus subtilis* by Graham (1930). It is certain that most of these authors were aware of the heat lability of the H antigen and were presumably selecting times and temperatures, which in their experience were safe. One is, however, impressed by the considerable variation of treatment, which would suggest either that the lability of H in the several species is not the same or that the reports are not comparable for reasons of technique.

There are a few papers which deal particularly with the effect of heat on the H substance. The early works of Joos (1903), Beyer and Reagh (1904), and Eisenberg and Volk (1902) are remembered only for recognizing that an antigen, which in later terminology became the H antigen, is heat labile. Joos, working with typhoid bacilli, described it as "rapidly destroyed at 60 to 62°C." Beyer and Reagh distinguished between impairment of agglutinability and effect upon antigenicity in the body; a temperature of 70°C. for 20 minutes accomplished the former but not the latter with the hog cholera bacillus. Smith and Ten-Broeck (1914-15) compared agglutinability of the fowl typhoid bacillus in suspensions unheated, and heated 1 hour at 60°C., 20 minutes at 65°C., and 20 minutes at 70°C. Only the last treatment caused appreciable injury. Orcutt in 1924 studied the flagellar antigen of the hog cholera bacillus originally used by Smith and Reagh. Both motile and non-motile strains were then available and a technique was devised for mechanically removing and separating flagella from their bacilli. Anti-flagellar serum was prepared with a titre of 1:320 for unheated flagella. With flagella heated for 20 minutes at 70°C. only a faint reaction, described as "fine" at 1:20 was given. From an absorption experiment it was found that power to combine with flagellar agglutinins was entirely lost by the heated flagella. They retained antigenicity *in vivo*, however.

Tulloch (1927) criticized the receptor analysis as revealing an "antigen-mosaic" structure of bacterial cells. He pointed out that the lability of the supposed H antigen is indefinite, depending upon whether the test is made *in vitro* or *in vivo*. Furthermore, "the degree of thermo-stability of a given reagent may not be

constant, and therefore, the terms thermo-stable and thermo-labile . . . are relative and arbitrary." He cited experiments in which the H antigen of a particular strain of *Salmonella aertrycke* apparently changed under laboratory cultivation. To show that 60°C. had no specificity in the inactivation of the H factor, he compared three strains of *Salmonella aertrycke* and one of *Salmonella paratyphi*, with antigens heated at 56°, 60° and 65°C. for 30 minutes. Whereas 56° to 60°C. seriously affected *S. aertrycke* as shown by altered agglutinability, *S. paratyphi* resisted 60° but was inactivated in 30 minutes at 65°C.

In other words, the point was well made that a specific and critical point of inactivation was not found. Thus, Tulloch was led to discard the whole concept of a particular labile antigen, and to suggest instead that the effect of heat "upon the organismal protoplasm is progressive and acts upon *all* components of the microörganism."

Opposed to this conclusion is the convincing demonstration by Craigie (1931) that heat causes *lysis* of the flagella and that coincident with their physical disintegration H agglutinability of a suspension of flagellated cells or separated flagella is lost. Moreover, the onset of lysis by heat was said to be sharp and an "essentially all or nothing phenomenon." Craigie therefore concluded that the flagella of the *Eberthella typhosa*, which he studied, "can be identified with the H receptor of Weil and Felix," or in other words, that the morphologically intact flagellum behaves as the H antigen. The reservation was made, however, that heat-lability of the flagellar antigen need imply only destruction by heat of the ability of the antigen to react in typical *in vitro* agglutination.

In considering the possible survival of H antigen as detected by other tests, Craigie applied the precipitin test and discovered that flagella lysed by heat (in treatments up to 2 hours at 100°C.) would still give unimpaired precipitation with pure flagellar agglutinating serum. This finding is of very great interest in relation to the occasional reports of unusual resistance of H antigen, as tested by *in vivo* reaction. But it also throws grave doubt on the validity of criteria of heat lability of the supposed H antigen.

It was to explore further the facts of heat inactivation of H antigen that the present work was undertaken. This paper will deal with the heat resistance of the H factors in two species, and the physical or chemical agents which may affect their apparent resistance. Interest in these species is increased by the fact that one is a mesophile and one a thermophile. The latter grows well at a temperature of 60° to 65°C. which is reported to injure, even in less than an hour, the H factor in species in the typhoid group, upon which the common impression of H lability is based.

#### EXPERIMENTAL

The cultures chosen were *Clostridium butyricum* (Piorowsky strain) and *Clostridium thermosaccharolyticum*. Both are motile species used by us in previous serological work (McClung and McCoy, 1935). For making antigen they were cultivated in 1 per cent tryptone—0.5 per cent glucose broth, the former at 37°C. for 24 hours, the latter at 60°C. for eighteen hours.<sup>2</sup> Five injections at 3-day intervals were given of cells living or heated as noted below, the dosage being graduated from 0.5 to 2 cc. of a suspension containing approximately 15 billion cells per cubic centimeter. Sera were taken from the fourth to seventh day after the last injection, tested with homologous antigen to demonstrate a satisfactory potency, and stored without preservative at 2° to 4°C.

After preliminary experiments it was decided to prepare sera for the untreated antigen (called W or whole-cell) and for antigens heated at two arbitrary degrees of temperature/time covering the probable range of inactivation. These were judged by *in vitro* tests in pure H antiserum (i.e., W serum pre-absorbed with O antigen); as (1) that highest temperature which in 30 minutes heating would *not* appreciably affect the agglutinability of the antigen, and (2) that temperature plus 5°C. for the same period.

<sup>2</sup> Inocula in all cases were taken from dry spore stock on sterilized soil and the actual cells collected for antigen for either animal injection or for the *in vitro* tests were those of the second culture generation from the spores. This statement is offered in part explanation of the remarkable constancy of agglutinability which we have been able to secure from experiment to experiment.

In the case of *C. butyricum* these treatments proved to be 60° and 65°C. for 30 minutes (*note* that these treatments are in the range of those debated in the literature); and for *C. thermosaccharolyticum* 80° and 85°C. respectively (*note* also that in no report on mesophiles has any claim been made of such resistance of H antigen). In certain experiments with *C. thermosaccharolyticum* use was made also of sera prepared from separated flagella by a method formerly described (McClung and McCoy, 1935).

The procedures of absorption, testing, and reading of results are essentially those of our previous work (McCoy and McClung, 1935). The experiments to be reported have been repeated two or more times with different sera and lots of antigens. It should be emphasized that all tests for survival of the H antigen are based upon readings of reactions in pure H antisera: i.e., W or flagellar sera completely O-absorbed to <1:40 by antigen which had been steamed 2 hours at 100°C. Any reactions then showing higher titers than 1:40 at least may be considered due to H reaction, irrespective of the quality of the flocculation. It should be emphasized that this precaution in distinguishing the H reaction has not been used previously.

*Evidence of in vivo antigenicity of untreated and heat-treated cells*

As a matter of course, the first points to be settled were the titers of the sera with their homologous antigens, and with known H and O antigen suspensions; in other words, the sera were analyzed for H antibody content. In tables 1 and 2 are given the data for the 3 types of sera for both the mesophile and the thermophile. By comparison of the series before and after absorptions, one is able to separate the H reactions from the H + O on a much more certain basis than by inspection of the *type* of flocculation. For example, in the case of the 85° serum for *C. thermosaccharolyticum* one would on the appearance of flocculation of W cells suppose the reaction to be of H type. But since the titer is the same for both W and O antigens, and since O absorption removes substantially all of the antibody in question one would finally conclude that the 85° serum after all contains no

**TABLE 1**  
*Analysis of antibody content of sera for Cl. butyricum*

SERUM FOR		TESTED WITH	SERUM DILUTIONS								
			80	160	320	640	1280	2560	5120	10240	
<i>Cl. butyricum</i> W			Direct								
			W	+++	+++	+++	+++	+++	+++±	+	—
			O	++	++	++	+	—	—	—	—
			60°	++	++	++	+	—	—	—	—
			65°	+	+	±	—	—	—	—	—
			After O absorption								
			W	+++	+++	+++	+++	+++	+++±	+	—
			O	—	—	—	—	—	—	—	—
			60°	++	++	+	—	—	—	—	—
			65°	—	—	—	—	—	—	—	—
60°C. 30 minutes			Direct								
			W	+++	+++	+++	+++	++	++	+	—
			O	+	+	+	+	—	—	—	—
			60°	++	++	++	±	+	—	—	—
			65°	+	+	+	±	—	—	—	—
			After O absorption								
			W	+++	+++	+++	+++	+++	+++	+++±	—
			O	—	—	—	—	—	—	—	—
			60°	++	++	+	—	—	—	—	—
			65°	—	—	—	—	—	—	—	—
65°C. 30 minutes			Direct								
			W	++	++	++	++	++	++	±	—
			O	+	+	+	—	—	—	—	—
			60°	++	++	+	+	—	—	—	—
			65°	±	±	—	—	—	—	—	—
			After O absorption								
			W	++	++	++	++	++	++	++±	—
			O	—	—	—	—	—	—	—	—
			60°	++	++	+	±	—	—	—	—
			65°	±	—	—	—	—	—	—	—

\* In this and other tables +++ indicates maximum agglutination; +++± etc., degrees of incompleteness; — negative.

W in all instances refers to the whole cell antigen, untreated by heat or chemicals. O refers to antigen prepared by steaming 2 hours at 100°C.

TABLE 2

*Analysis of antibody content of sera for Cl. thermosaccharolyticum*

SERUM FOR	TESTED WITH	SERUM DILUTIONS								
		80	180	320	640	1280	2560	5120	10240	20480
<i>Cl. ther.</i> W	Direct									
	W	+++	+++	+++	+++	+++	++±	++	-	-
	O	++	++	++	++	++	++	+	-	-
	80°	++	++	++	++	+±	±	±	-	-
	85°	+	+±	+±	+	+	+	±	-	-
	After O absorption									
	W	+++	+++	+++	+++	+++	+++	++	-	-
	O	+	-	-	-	-	-	-	-	-
	80°	+	+	+	+	-	-	-	-	-
	85°	±	±	-	-	-	-	-	-	-
80°C. 30 minutes	Direct									
	W	+++	+++	+++	+++	+++	+++	+++	+++	+
	O	++	++	++	++	++	++	++	++	+
	80°	++	++	++	++	++	+	-	-	-
	85°	±	+±	+±	+±	+±	+	+	-	-
	After O absorption									
	W	+++	+++	+++	+++	+++	+++	+++	+	+
	O	+	-	-	-	-	-	-	-	-
	80°	+	+	-	-	-	-	-	-	-
	85°	+	-	-	-	-	-	-	-	-
85°C. 30 minutes	Direct									
	W	+++	+++	+++	++±	+	+	-	-	-
	O	++	++	++	++	++	+	-	-	-
	80°	+	++	++	++	+	-	-	-	-
	85°	+	+	+	+	+	+	-	-	-
	After O absorption									
	W	+	+	-	-	-	-	-	-	-
	O	+	-	-	-	-	-	-	-	-
	80°	-	-	-	-	-	-	-	-	-
	85°	+	-	-	-	-	-	-	-	-

detectable H antibody. In a similar way the table shows that the heat-treated antigens in all cases react less completely, and generally in a range of dilution within the O titer of the serum. It is only after absorption that one can see the ability of the 60° and 80° antigens to react poorly but positively to H antibody, whereas the 65° and 85° antigens react very slightly or not at all. It should perhaps be noted that our arbitrary choice of temperatures for the 30-minute pretreatment of the antigens for injection was successful only in the case of the thermophile, in that the 80° antigen was still H-antigenic *in vivo*, the 85° not. This point is to be noted in relation to the contention of Craigie and others that *in vivo* antigenicity survives heat treatments greatly in excess of *in vitro* reactivity. That the 65° cells of *C. butyricum* were not inactivated so completely is not surprising, since the rate of inactivation at the range of 60° and 65°C. should be considerably less than between 80° and 85°C. In any case, however, the prospect would be that 100°C. for 2 hours, which we have used for H inactivation in producing O suspensions, would be safely in excess of the heat resistance of the H factor of either species.

*Evidence by in vitro tests of the course of heat inactivation*

If Craigie's proposal that heat affects the H antigen by causing lysis of the flagella is correct, one might find a critical temperature at which all flagella would be destroyed. Obviously *time* of exposure would be a factor, and so, as in experiments on the thermal death time of organisms, it is only necessary to choose a temperature calculated to be effective in a convenient time for the experiment, and to plot a curve of time against titer of the H antigen as shown by *in vitro* agglutinability. Any sharp break in the curve would correlate with lysis. This type of experiment was used by Tulloch, but only in a limited way with exposures of 5, 15, and 30 minutes at 60°C. Perhaps because his sera were not pure anti-H (and were possibly complicated by group- and specific-phase reactions) his results were not clear cut and were in fact used to support his argument for the progressive inactivation by heat of all components of the cell.

In the present work experiments along these lines were found

very useful. It is from such curves of heat inactivation that one can compare the behavior of the mesophilic and thermophilic H antigens. One can also try the effect of chemical and physical factors and thereby come to understand how in the literature seemingly erratic variations are reported. A few experiments will illustrate.

Figures 1 and 2 compare the inactivation curves at 3 temperatures, the times of treatment being from 0 to 60 min.; the experi-

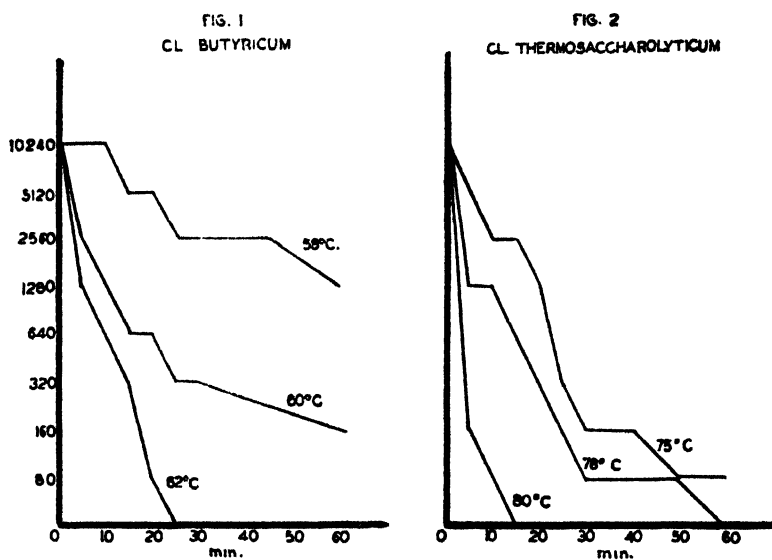


FIG. 1. CURVES OF INACTIVATION OF THE H AGGLUTINOGEN OF *CL. BUTYRICUM* AT 58°, 60°, AND 62°C.

FIG. 2. SIMILAR CURVES FOR *CL. THERMOSACCHAROLYTICUM* AT 75°, 78°, AND 80°C.

ment was done with serum O-absorbed to <1:40 with somatic antigen, so that the curves represent H inactivation only. It is evident that destruction of H is a time/temperature function and that small differences in temperature, like 2°C., make a great difference in the rate of inactivation. In our own early experiments, when temperatures were not accurately controlled somewhat erratic results were obtained in some cases, and it seems to us very likely that some of the confusion in the literature results



from lack of appreciation of this point.<sup>3</sup> It is evident also from the figures, by comparison of results for the two species, mesophilic and thermophilic, that the differences in slope are less than would be expected considering the different range of temperatures at which the curves are drawn. This is better illustrated in figure 3, a comparison of the 60° curve for *C. butyricum* with an 80° curve for *C. thermosaccharolyticum*. The essential

FIG. 3

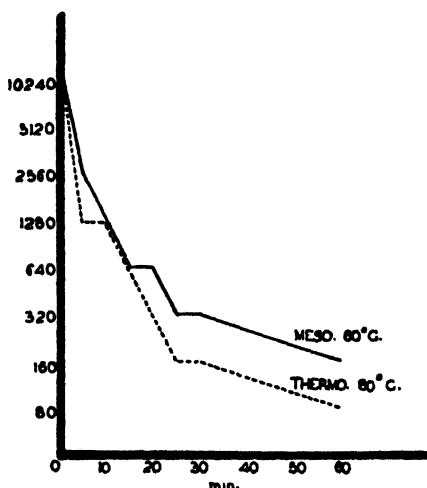


FIG. 3. COMPARISON OF THE HEAT RESISTANCE OF THE MESOPHILIC AND THERMOPHILIC AGGLUTINOGENS

likeness of the two curves would seem to mean that the H factor of the thermophile is inherently more resistant (or is so protected in the cell as to appear so). In no case in these curves is there a

<sup>3</sup> The desired amount, up to 10 cc., of fresh concentrated stock antigen was placed in a test tube together with a 1 cc. pipette and a standard thermometer. The tube was placed in a boiling waterbath to bring the temperature as quickly as possible to the desired degree; 1 cc. was immediately removed by pipette to make the "zero minute" diluted antigen, and the tube transferred to another deep waterbath at the desired degree of temperature. With constant stirring both in the tube and in the waterbath the temperature was maintained with great care and 1 cc. portions taken every 5 minutes up to 30 minutes and beyond that at 10- to 15-minute intervals as needed for particular experiments. Because the results have been reproducible from experiment to experiment, we believe that temperature has been held with sufficient accuracy.

sign of sharp change of slope as one would expect if there were a critical point of lysis of the flagella. Rather do the curves suggest a progressive change such as coagulation for the mechanism of inactivation. They closely resemble death curves in disinfection or thermal death times of bacterial populations, and do, in fact, plot to straight line logarithmic curves.

Another use of the time curves has been to demonstrate the effect of one or another chemical factor upon the rate of inactivation by heat. A series of experiments was planned to show the effect of pH 5 to 9, with antigens heated to 58° and 60° and 75° and 78°C. for the mesophile and thermophile respectively. For

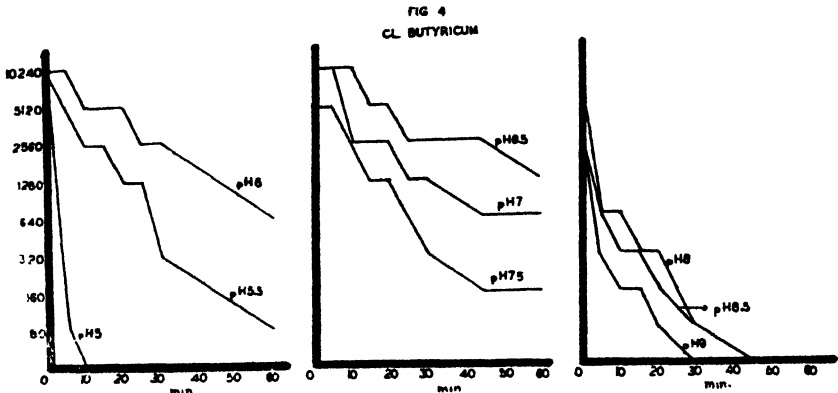


FIG. 4. EFFECT OF pH ON THE APPARENT RESISTANCE OF THE H AGGLUTININ OF CL. BUTYRICUM

the antigens in these experiments, cells were collected in the usual way by centrifugation and resuspension in normal saline in concentration so that *after* heating about 1 to 25 dilution gave the desired antigen for test. Colorimetric pH indicators were added to suitable portions and the desired pH obtained by addition of HCl or NaOH as needed. Control experiments were included to show that the presence of the indicators during heating and subsequent agglutination tests had no unfavorable effects. Control on the effect of pH apart from heat was also provided in a series held in the ice box for 24 hrs. Since this treatment did not change the titers appreciably from those of zero minutes at 60° or 75°C. the data need not be presented here. Figures 4 and 5 present

the set of curves for the series heated at 60° and 75°C., for the mesophile and thermophile respectively. It is evident from either series that pH has a profound effect upon the rate of inactivation of the H factor by heat. As might be expected the steepest curves are at the extremes of pH tested; in fact, in the case of *C. thermosaccharolyticum* complete destruction in less than 5 minutes at pH 8.5 to 9 has prevented drawing of curves at all. The pH of least destruction differs slightly for the two species, but in both cases falls in a slightly acid range. The point should be made that the absolute amount of acid required to adjust the lots of antigen even to pH 5 was not great, because of the absence

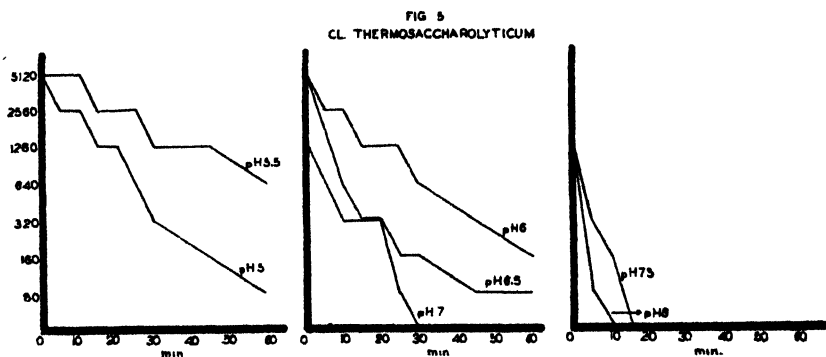


FIG. 5. SIMILAR pH CURVES FOR CL. THERMOSACCHAROLYTICUM

of buffer other than the protein of the cells in the saline suspension of concentrated antigen; N/10 acid was added, drop by drop, and often only 3 to 5 drops per 10 cc. of suspension were required. These points become significant when one considers the recent tendency to recommend use of glucose broth cultures directly (Weinberg and Barotte, 1928, and Hall, 1937) for both immunization and performance of agglutination tests with anaerobes. The pH of such glucose cultures may well be at or below 5 (see Snyder, 1936), and if such antigenic suspensions are then treated even at 56° to 60°C. for 1 hour to inactivate toxin, very serious injury of the H agglutinin will have been produced.

Experiments were made on the effect of two other factors, which by analogy to their protective effect on thermal death times

of bacteria appeared likely to modify the rate of inactivation of H antigen by heat. In one experiment normal blood serum was added to serve as a protective colloid, in concentration of one-third by volume in the concentrated antigen heated. Series were carried out at pH 6 and 7 in order that acid coagulation of the serum proteins might not be a complicating factor; for that reason also the experiment has been done only with *C. butyricum* at 58°C. No flocculation during heating did occur and apparently, from figure 6, no protective action was exerted in comparison with control series run at the same time in saline alone.

FIG. 6

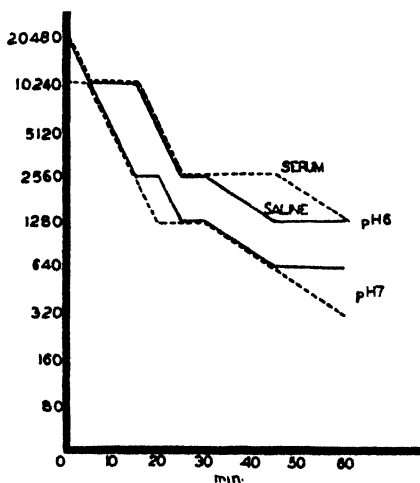


FIG. 6. RESISTANCE OF THE H AGGLUTINOGEN OF *C. BUTYRICUM* HEATED IN SALINE AND IN THE PRESENCE OF SERUM PROTEINS

Another experiment was set up in which a dehydrating agent was tested for its effect upon the survival of H antigen. For this purpose glycerine was chosen because it is known to have no inhibitory effect upon agglutination, often being used in serum preservation for this reason. In the present experiment 50 per cent by volume of glycerine was used in the concentrated antigen heated. Because it was found to have a decided protective action a series of tests had to be run to produce effects within the range of time of experiment. Figure 7 illustrates three of the glycerine

series, together with their saline controls. The protective effect of the glycerine is remarkable.

Variations in the concentration of sodium chloride were also tried. The serum for these experiments was absorbed with a concentrated O-antigen in 0.4 per cent saline and diluted to a saline concentration of 0.1 per cent after addition of the last absorbing dose. It was then possible to run series in the range from 0.1 to 2 per cent by arranging to dilute each set in the desired grade of saline, the 0.1 per cent NaCl in the absorbed

FIG 7

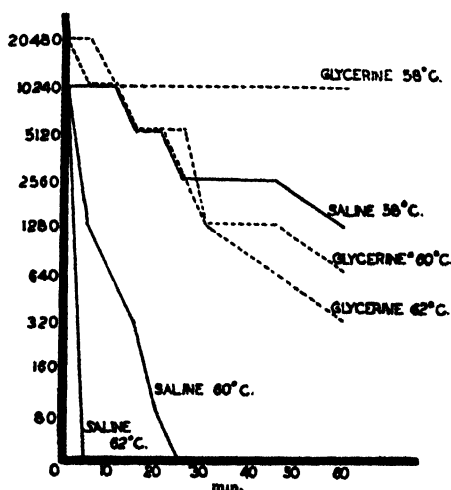


FIG. 7. EFFECT OF GLYCERINE AS DEHYDRATING AGENT ON THE RESISTANCE OF THE H AGGLUTINOGEN OF *CL. BUTYRICUM*

serum being taken into account. The antigens were made to the proper cell dilution for the tests in saline of the same concentrations, so that it was possible to carry out both heating and test after heating in the same saline concentration. An arbitrary time and temperature of heating were chosen for these experiments and the data are given in table 3. There is very definitely an optimum concentration for agglutinability of the unheated antigen, and, curiously the same concentration is best for survival of that antigen to heat in the treatment given. It is perhaps

understandable that the lowest concentration tested, 0.1 per cent, should be most destructive to flagella and consequently to H, for it is most hypotonic. But that 1.5 and 2 per cent should be

TABLE 3

*Effect of concentration of NaCl upon the survival of H antigen*

ANTIGEN	NaCl %	SERUM DILUTIONS							
		80	160	320	640	1280	2560	5120	10240
<i>Cl. butyricum</i>									
Unheated controls	0 1	++	++	++	++	++	++	++	+
	0 2	++	++	++	++	++	++	++	+
	0 4	+++	+++	+++	+++	+++	++	++	+
	0 8	+++	+++	+++	+++	+++	+++±	++	+
	1 5	++	++	++	++	++	++	+	-
	2 0	++	++	++	++	++	++	+	-
Heated 60° 10 minutes	0 1	-	-	-	-	-	-	-	-
	0 2	++	++	++	+	+	±	-	-
	0 4	+++	+++	+++	+++	++	++	+	-
	0 8	+++	+++	+++	+++	+++	+++±	+	-
	1 5	-	-	-	-	-	-	-	-
	2 0	-	-	-	-	-	-	-	-
<i>Cl. thermosaccharolyticum</i>									
Unheated controls	0.1	+	++	++	++	++	++	+	-
	0.2	+	++	++	++	++	++	±	-
	0 4	+++	+++	+++	+++	+++	+++	+	-
	0.8	+++	+++	+++	+++	+++	+++	+	-
	1 5	+	+	+	+	+	+	-	-
	2 0	+	+	+	+	+	-	-	-
Heated 75° 10 minutes	0.1	-	-	-	-	-	-	-	-
	0 2	+	+	+	+	-	-	-	-
	0 4	+++	+++	+++	+++	+++±	+	-	-
	0 8	+++	+++	+++	+++	++	+	-	-
	1.5	-	-	-	-	-	-	-	-
	2 0	-	-	-	-	-	-	-	-

even more injurious is more difficult to explain. Fortunately 0.8 or 0.4 per cent are used in most agglutination work; and it may be said that in all of the preceding experiments in this paper 0.8 per cent was employed.

*Evidence by tests of absorption capacity of heat-treated cells*

In view of the apparent discrepancy between heat inactivation as judged by H agglutinability and by *in vivo* antigenicity, some

TABLE 4

*Absorption tests with W serum for Cl. thermosaccharolyticum*  
(Absorptions by W, O and antigens heated at 78° to 86° for 30 minutes and 100° for 10 minutes)

SERUM FOR	ABSORBED BY	TESTED WITH	SERUM DILUTIONS						
			80	160	320	640	1280	2560	5120
W	O	O	..	..	..	..	..	..	..
		W	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
		78°	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
		80°	XXX	..	..	..	..	..	..
		82°	..	..	..	..	..	..	..
		84°	..	..	..	..	..	..	..
		86°	..	..	..	..	..	..	..
		100°	..	..	..	..	..	..	..
W	W	O	XXX	..	..	..	..	..	..
		W	XXX	..	..	..	..	..	..
		78°	XXX	..	..	..	..	..	..
		80°	XXX	..	..	..	..	..	..
		82°	..	..	..	..	..	..	..
		84°	XXX	..	..	..	..	..	..
		86°	..	..	..	..	..	..	..
		100°	..	..	..	..	..	..	..
W	78°	O	..	..	..	..	..	..	..
		W	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
		78°	XXXXXXXXXXXXXXXXXXXX	..	..	..	..	..	..
		80°	XXX	..	..	..	..	..	..
		82°	..	..	..	..	..	..	..
		84°	..	..	..	..	..	..	..
		86°	..	..	..	..	..	..	..
		100°	..	..	..	..	..	..	..
W	80°	O	..	..	..	..	..	..	..
		W	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
		82°	XXXXXXXXXXXXXXXXXXXX	..	..	..	..	..	..
		80°	..	..	..	..	..	..	..
		82°	..	..	..	..	..	..	..
		84°	..	..	..	..	..	..	..
		86°	..	..	..	..	..	..	..
		100°	..	..	..	..	..	..	..





work was needed on another test of survival of the H antigen. In other words, the third criterion of antigenic function, absorptive capacity, remained to be tested, and it was of particular interest to see whether its inactivation would parallel that of agglutinability or agglutinin production. For this purpose the sera for *C. thermosaccharolyticum* were chosen, because they represent a series covering whole-cell antiserum, one for cells heat-treated so as to injure agglutinability but not antigenicity *in vivo*, and one for cells heat-treated so as to destroy so far as the first tests indicated all H antigenic behavior.

TABLE 6

Absorption by pH 5, 6, 8, and 9 antigens after heat treatment of 60 minutes at 75°C.  
(*Cl. thermosaccharolyticum*)  
(Serum—anti W)

ABSORBED BY		TESTED WITH	SERUM DILUTIONS						
			80	160	320	640	1280	2560	5120
Antigen heated at:	pH 5	O							
	75°—60 minutes	W	XX						
	pH 6	O							
	75°—60 minutes	W	XX						
	pH 8	O							
	75°—60 minutes	W	XX						
	pH 9	O							
	75°—60 minutes	W	XX						

Tables 4 and 5 contain the data. Naturally the W antigen in any serum exhibits H-absorbing capacity and incidentally shows again that serum for 85° antigen is devoid of H agglutinins. The only heat-treated antigen to show *any* H absorbing action is the 78° antigen and that is slight. That 78° antigen also reacts slightly wherever H antibody is left after absorption is in line with expectation. The remaining antigens heated at 80°, 82°, 84°, 86° for 30 minutes and 100° for 10 minutes behave as *somatic absorbing agents only*. This consistent evidence of complete inactivation of H absorbing capacity is of particular interest,

because it corresponds exactly with ability/inability to agglutinate (see figures 1 and 2 again: antigen 78° in the state after heating 30 minutes is the last to show appreciable reaction).

One further test was made for the absorptive capacity of antigens heat-treated under conditions to destroy their H agglutinability. Antigens from the extremes of the pH series, i.e., pH 5, 6, 8, and 9, heated to 75° for 60 minutes were known from former tests to have agglutinability reduced to about 1:80. Their reactions were adjusted back to that known to be harmless in absorption tests, pH 6.0 to 6.5, and they were then used as absorbing agents in the usual way. Table 6 shows that they had no more absorbing capacity than they had agglutinability.

From the above experiments it would seem that ability to absorb H agglutinins closely parallels ability to agglutinate to the same.

#### SUMMARY

It is evident that the inactivation by heat of the H agglutinin of bacteria is a complex phenomenon, and is not to be defined in terms of an arbitrary temperature and time. Factors such as the acidity or alkalinity of the suspending medium, the presence of a strong dehydrating agent like glycerine, and to some extent of even low concentrations of NaCl have profound effect upon the apparent heat resistance of the H factor. It is probable that protective colloids in the medium would also be important, although serum proteins did not appear to be effective under the conditions tested.

Granted that the apparent heat resistance of the H antigen can be manipulated over a considerable range, what becomes of the concept of heat lability as defining the H factor? One must, it seems, agree with Tulloch that the terms, "thermostable and thermo-labile . . . are relative and arbitrary." But one need not assume for that reason that no distinction of antigens can be made by the application of heat or, as Tulloch has put it, that the change upon heating is progressive alteration of "all components of the organismal protoplasm." If that were true, there would hardly be the discrepancy between the highest known resistance

of a so-called H factor and the much higher (as yet undetermined maximum) resistance of the somatic factors of the same species. We submit that the disappearance, as judged by all three criteria of antigenicity, of the H agglutinin of *Clostridium thermosaccharolyticum* heated at 85°C. for 30 minutes is evidence on this point.

Furthermore, if the effects of heating were not differential for antigens, there would not be the great difference in range of treatment for inactivation of the corresponding factors in the mesophilic and thermophilic species. The likeness of behavior of their H agglutinogens has been remarkable, but with the important difference that the antigens in question are 20°C. apart in lability. Twenty degrees for the times employed is far more than the range over which the heat resistance of either H antigen can be manipulated by the influence of the chemical or physical factors so far investigated.

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# ON LEEUWENHOEK'S METHOD OF SEEING BACTERIA

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In his famed letter of October 9th, 1676, to the Royal Society of London, Leeuwenhoek wrote: "My method for seeing the very smallest animalcules and minute eels, I do not impart to others; nor how to see very many animalcules at one time. That I keep for myself alone." His trick of manipulation has remained something of a mystery; although Robert Hooke, who independently repeated some of the observations, seems to have left no record of any unusual technique. Clifford Dobell (1932) has expressed a conviction that the pioneer microscopist must have hit upon some method of dark-field illumination.

It will be recalled that Leeuwenhoek's "microscope" was a single, biconvex magnifying glass which he ground and then mounted between small apertures in a metal holder. The instrument was held close to the eye and the object on the other side of the lens was adjusted to proper focus. The fluids to be observed were usually held in thin glass capillary tubes. With this apparatus, he obtained sufficient contrast between floating bacteria and their background to see and describe them, often recognizably.

The following experiments may perhaps throw some light on Leeuwenhoek's procedure. The first series was done with fused glass spheres about 1.6 mm. in diameter, set into bevelled 0.9 mm. apertures in sheet copper. Their constants, calculated approximately, are: magnification, 230; working distance, 0.3 mm.; numerical aperture, 0.4. These simple lenses have amazingly high power, for they can resolve, without detectable distortion, the rulings of a Wallace replica grating with 25,000 lines to the

inch. In a hanging drop upon a cover-glass, red blood cells can be easily observed with them, and small bacteria like *Escherichia coli*, after a little practice. It was more difficult, but nevertheless possible, to see the unstained bacteria in fine capillary tubes. As van Cittert (1933) points out, a measure of dark-field illumination occurs at the meniscus in the capillary, but I was unable to observe the bacteria in this region. In my observations, direct light was used, either from a microscope lamp or from the north sky.

The second group of experiments was the result of a discussion with Prof. E. G. Hastings<sup>1</sup> and Prof. E. B. Fred of the University of Wisconsin, who suggested that the curvature of a drop of fluid should give the effect of an additional lens (thereby adding to the magnification of objects suspended within it), and further, that the droplet might produce a dark-field effect under proper illumination.

Attempts to test these ideas upon bare drops of cell suspension met with indifferent success, because manipulation was necessarily slow, and the small drops evaporated and changed in curvature too rapidly to permit confident observation. This difficulty might be controlled in a room of high relative humidity, but such a room was not available; moreover, the experimental conditions were unattractive and probably not comparable to those under which Leeuwenhoek worked. It might be possible also, as Professor Hastings suggests, to feed a hanging drop from a capillary tube and thus to compensate for the evaporation.

However, there was an obvious and more practicable alternative in covering the droplet to protect it from evaporation and to preserve its shape. This was accomplished by placing the fluid in a spherical bulb, 1 to 3 mm. in diameter, blown on the end of a fine capillary tube. A number of these were made and filled

<sup>1</sup> I am indebted to Professor Hastings for submitting the results of the preliminary experiments which he had performed alone, and in collaboration with Professor Fred and the late Professor Mendenhall. Dr. Hastings states that he was able to show that yeast cells could be seen in a drop of water suspended from a support, with magnifications which did not reveal the cells unless the drop itself had some rôle in the magnification.

with pepper-infusion, or suspensions of red cells or bacteria. Examination of these preparations with the fused glass spheres mentioned above confirmed the first observations. A small degree of additional magnification (about  $1.5\times$ ) was observed, especially in the smallest bulbs. Moreover, rather good dark-field effects were obtained by inspecting the bulbs under suitable lateral illumination.

I have used also a copy of a Leeuwenhoek microscope having a small lens (presumably unground, like the above) before which was adjusted one of the spherical bulbs containing a cell suspension. Red cells, bacteria and minute particles were seen with good definition on frontal illumination, but the observations were rather arduous. As to this, Leeuwenhoek said: "and in the close inspection of 3 or 4 drops, I may indeed expend so much labor that the sweat breaks out on me." Under lateral illumination, striking dark-field views were obtained. Additional magnification, of the order of  $2\times$ , was secured by taking advantage of the following fact. When a bubble of air is introduced into a spherical body of water, particles in the proximal air-water surface are magnified, while those in the distal surface are diminished in apparent size. A few air-bubbles were therefore admitted into the bulb and focus was directed upon the material collected in the near surface of one of them. The cells were not only magnified there, but they remained in a relatively fixed position with reference to the lens,—an appreciable aid in the troublesome manipulative details. By working in a dark-room and employing lateral illumination with proper shading, this procedure enabled me finally to see red cells well magnified in dark-field view, "like sand-grains on black taffeta."

In my last group of experiments, observations were tried with the lens *immersed* in the cell suspensions. This was accomplished by first tracing a ring of vaseline around the rim of the lens aperture to prevent seepage of fluid, and then depositing a large (3 mm.) drop of suspension centrally over the lens and its frame. After some practice, it was possible to see red cells and bacterial masses within the drop rather clearly. Visualization of single bacteria was also obtained, but this required considerable effort.



However, on observing the fluid as it slowly evaporated down, there came into sharp focus the surface of the drop and the myriads of cells and particles collected in that region. An approach to dark-field illumination was also obtained by the immersion method, but this was not investigated further. It is obvious, of course, that immersion increases the effective magnification and resolution of the lens.

In summary, it may be stated that one can augment the effectiveness of a simple lens by suitably utilizing the inherent optical properties of the spherical drop of fluid containing the objects under observation. The advantages of a water-immersion objective are too well known to require comment; but the added advantage of what amounts to the superposition of a relatively thick meniscus lens (of water) may be worthy of mention. There is apparently no way to prove that Leeuwenhoek did actually employ either of the simple devices set forth above; but certainly, their production was well within the facilities and competence of that clever manipulator. At any rate, the experiments described herein offer at least two plausible explanations of the secret method that he so jealously guarded.

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# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## CENTRAL NEW YORK STATE BRANCH

33RD SEMI-ANNUAL MEETING, GENEVA, NEW YORK, JUNE 5, 1937

**INFLUENCE OF DRYING UPON SOIL USED AS A MEDIUM FOR BACTERIA.** *H. J. Conn and Mary Darrow*, New York State Agricultural Experiment Station, Geneva, New York.

*Bacterium radiobacter* has been inoculated into sterilized soil, after bringing it to optimum moisture content and adding nutrients sufficient to assure good growth of the organism. Its numbers have then been determined by use of the microscope. Under optimum conditions the counts reach about two billions per gram. In some soil samples, however, it was found that no matter what nutrients were added the organism was not able to multiply to much over one billion, if it even reached that figure. Some unfavorable condition seemed to be holding it in check.

As the soils in which its members remained low had all been for some time in air-dry condition, the effect of varying moisture content was studied. This study has now been made on three different soils, and it has been found that keeping them moist for about two weeks before adding the nutrients and sterilizing does improve them as a medium for the growth of this organism. Best results were obtained when the soil was kept for this period moistened to about 30 to 35 per cent of its water-holding capacity. After two weeks at this moisture content, however, these soils become less favorable

for the organism. No explanation of the phenomenon is yet offered.

**PAPER MILL SANITATION IN RELATION TO THE MANUFACTURE OF FOOD WRAPS AND CONTAINERS.** *J. R. Sanborn*, New York State Agricultural Experiment Station, Geneva, New York.

The making of paper containers for such a perishable and easily contaminated product as milk gives paper manufacture increased public health significance. Certain types of container board are heavily contaminated with coliform organisms; in other cases, spore-bearing bacteria, micrococci, or filamentous fungi may predominate.

Uncontrolled development of microorganisms in pulp and paper mills presents serious obstacles to quality in paper wrappers and containers. Accumulations of growth as stringy or gummy masses interfere with production and result in spotting and in lack of uniformity and strength. Discoloration and decomposition processes in stored pulp or stock definitely affect quality, as well as the presence of objectionable odors, which are sometimes transferred from pulp to finished paper.

Strict microbiological control of pulp and paper operations is successful in preventing such difficulties and also sets a sanitary standard for plants engaged in fabrication and handling of food wraps and containers. Programs

for control include general sanitary practices, systematic slime prevention measures, chemical treatment of process water designed to reduce bacterial numbers in pulp, and handling of sheets so as to render them suitable for direct contact with such foods as milk and meat. Wrapper and container board for foods should, upon leaving a mill, conform to sanitary and bacteriological standards.

**PREVALENCE OF HUMAN INFECTION WITH TRICHINELLA SPIRALIS.** *O. R. McCoy*, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

**A MODIFICATION OF THE FIBRINOLYTIC TEST FOR STREPTOCOCCI.** *J. M. Sherman, F. R. Smith and C. F. Nivan*, Laboratory of Bacteriology, Cornell University, Ithaca, New York.

**RECENT ATTEMPTS TO STABILIZE BACTERIOLOGICAL NOMENCLATURE.** *R. S. Breed*, New York State Agricultural Experiment Station, Geneva, New York.

**AN EVALUATION OF FORMATE-RICINOLEATE BROTH FOR THE DETECTION OF COLON ORGANISMS IN RAW AND PASTEURIZED MILK.** *I. C. Gunsalus and C. N. Stark*, Laboratory of Bacteriology, Cornell University, Ithaca, New York.

Six hundred fifty-five samples of pasteurized milk from various parts of New York State during different seasons have been tested in formate-ricinoleate broth for the presence of members of the *Escherichia-Aerobacter* genera. Of these, 147 gave positive presumptive results, 144 of which confirmed according to Standard Methods. Two

of the three unconfirmed samples yielded slow lactose-fermenting members of the *Escherichia* genus, and the third a related species which belonged to the *Escherichia* or *Salmonella* genus.

In comparative tests on 221 samples of pasteurized milk, formate-ricinoleate broth, brilliant green 2 per cent bile broth, enrichment in standard lactose broth (24 hours' incubation) followed by inoculation into formate-ricinoleate broth, enrichment in buffered lactose broth (24 hours' incubation) followed by inoculation into formate-ricinoleate broth, gave approximately the same number of positive presumptive tests. A slightly higher yield of positive presumptive tests was indicated for the brilliant green bile broth, but some of these were due to anaerobic spore producing rods.

Of 99 samples of raw milk tested in formate-ricinoleate broth, 56 gave positive presumptive tests. Of these, 47 confirmed according to Standard Methods. From five of the nine sample which did not confirm slow lactose-fermenting *Aerobacter* organisms were isolated; from the other four samples *Proteus* organisms which were able to produce gas from formate, were isolated.

Fifty-three organisms which, according to Standard Methods, yield false presumptive tests were found to belong to the *Proteus Aerobacter* and *Escherichia* and related groups

**CITRATE-RICINOLEATE AGAR FOR THE DETECTION OF ESCHERICHIA, AEROBACTER, AND PROTEOLYTIC GRAM-NEGATIVE RODS IN MILK.** *M. L. Littman and C. N. Stark*, Laboratory of Bacteriology, Cornell University, Ithaca, New York.

The composition of the citrate-ricinoleate agar used was: 0.5 per cent peptone; 0.1 per cent sodium ricino-

leate; 0.4 per cent sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2} \text{H}_2\text{O}$ ); 0.2 per cent sodium nitrate; 1.5 per cent agar; neutral red 1/20,000; and brom thymol blue 1/20,000. The pH was 7.0. The 1 cc. inoculations of milk add to the citrate ricinoleate agar approximately 0.5 per cent lactose.

The ability of sodium ricinoleate to inhibit the growth of organisms associated with false tests was demonstrated in this medium. Sodium nitrate is used to prevent the production of gas which would cause explosion of the agar and make accurate counting of colonies difficult. *Aerobacter* and proteolytic organisms produce an alkaline reaction from sodium nitrate, but this salt is not used as the sole source of carbon. On this medium containing neutral red, *Escherichia* organisms, able to produce acid from lactose, but unable to attack citrate, appear as large red colonies. *Aerobacter* organisms, able to attack citrate, show an alkaline reaction, signified in the presence of brom thymol blue by the production of a blue green color. Proteolytic organisms can be detected on this medium, containing 10 per cent milk, by the presence of a clear zone around the colonies. These zones may be made more conspicuous by flooding the plates with an acid solution of bichloride of mercury or another similar substance.

Plating milk samples of citrate-ricinoleate agar makes possible the differentiation and enumeration of *Escherichia*, *Aerobacter*, and proteolytic Gram-negative rods.

**A COMPARISON OF BRILLIANT GREEN LACTOSE BILE AND FORMATE RICINOLEATE MEDIA FOR THE DETECTION OF THE *ESCHERICHIA-AEROBACTER* GROUP IN MILK AND ICE CREAM.**  
*H. W. Leahy, Rochester Health Bu-*

*reau Laboratories, University of Rochester, Rochester, New York.*

The relative effectiveness of brilliant green lactose bile broth 2 per cent and of formate-ricinoleate broth for the routine detection of the *Escherichia-Aerobacter* group in pasteurized milk and ice cream has been studied. Of 542 samples (369 milk and 173 ice cream), 219 gave positive presumptive tests in each medium; of these, 197 were confirmed from brilliant green lactose bile broth and 192 from formate-ricinoleate broth. The per cent confirmation, therefore, was 90 per cent and 88 per cent respectively. An analysis of these data was made by an adaption of Halvorson and Ziegler's method. It showed that the two media were equally useful for the routine detection of the *Escherichia-Aerobacter* group in pasteurized milk and ice cream.

**RABBIT FIBROMA TO MYXOMA TRANSFORMATION WITH HEAT-INACTIVATED MYXOMA ELEMENTARY BODIES.**  
*George P. Berry, Department of Bacteriology, University of Rochester, School of Medicine and Dentistry, Rochester, New York.*

Experiments from our laboratory have been reported as showing that the virus of Rabbit Fibroma (Shope) can be changed into that of Infectious Myxomatosis (Sanarelli). In these transformation experiments, rabbits were inoculated with suitable mixtures composed of active fibroma virus and heat-inactivated myxoma virus, the latter being present in heavy suspensions of myxomatous skin lesions. Besides myxoma virus, these crude suspensions obviously contained a host of other things. It was possible, therefore, that the "transforming agent" might not have come from the virus, but from the infected host. The work

described below not only indicates that the "transforming agent" is derived from myxoma virus, but also adds strong confirmation to our interpretation that fibroma virus is actually changed into myxoma virus. Through the kindness of Dr. T. M. Rivers, we have been able to work with suspensions of washed elementary bodies of myxoma, prepared in his laboratory at the Rockefeller Institute. These suspensions are essentially free of rabbit tissue and produce myxomatosis in a

dilution of 10<sup>7</sup>. Heating them at 65°C. for 30 minutes renders them completely non-infectious. In 5 experiments with 3 different myxoma elementary body suspensions, inactivated at 65°C. and at 75°C., we have transformed fibroma virus into myxoma virus.

FACTORS INFLUENCING THE RATE OF LACTIC FERMENTATION. *Otto Rahn, C. P. Hegarty and E. P. Deuel*, The Laboratory of Bacteriology, Cornell University, Ithaca, New York.

### EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND TWENTY-FIFTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PENNSYLVANIA, MAY 25, 1937

BACTERIAL TYPE TRANSFORMATION.  
*Hobart A. Reimann*, Jefferson Hospital, Philadelphia.

Bacterial type transformation has been discussed for years in regard to many bacteria and viruses. The theoretical importance of type transformation in regard to infectious disease and to epidemiology is obvious.

In 1934 a strain of *Micrococcus tetragenus* was obtained from the blood of a patient with meningitis and arthritis. The organism produced white colonies which, upon aging, developed yellow daughter colonies and a third or translucent form. Subsequently pink, pink-yellow and brown colonies appeared, each of which bred true. They were immunologically distinct types. Spontaneous transformation from one type into another was repeatedly observed, it being an apparent chance phenomenon rather than forced variation. Each type was dissociated into its M, S and R culture-phases; 15 variants of the original white form being isolated and studied.

The various forms exhibited constant differential cultural preferences for different ranges of temperature and

hydrogen-ion concentration of media, and different degrees of resistance to various bactericidal influences. The white form, as derived from the patient, grew best at temperature, CO<sub>2</sub> tension and pH ranges similar to those found *in vivo*. It appeared that type transformation and culture-phase variation were phenomena which permitted a bacterium to exist in a wider range of environmental conditions.

THE EFFECT OF DIETARY MINERALS UPON HOST RESISTANCE. *Charles F. Church*, Department of Pediatrics, School of Medicine, University of Pennsylvania and Children's Hospital of Philadelphia.

The effect of diet upon host resistance has been tested in 2000 mice of three inbred lines. The animals were inoculated by stomach tube with 0.005 cc. of an 18-hour broth culture of *Salmonella enteritidis* (3 to 5 million organisms). The stock culture used throughout the work has been kept on agar slants in the ice-box and has shown no change in virulence.

Groups of 20 mice of the same genetic and dietary background were compared

on each experimental diet. The survival per cent at four weeks after inoculation was taken as the index of host resistance.

Five groups of Line A (Rockefeller Institute Resistant) mice on the standard purified diet showed a mean survival of 91.0 per cent  $\pm$  2.9 (standard deviation). When the mineral content of the diet was reduced to one-fourth that of the standard, leaving all other factors unchanged, the survival in five groups of A-line mice was 67.5 per cent  $\pm$  4.6. The odds against this difference being the result of chance are 20,000:1.

When calcium only was reduced in the diet (from 149 mgm. per 100 calories to 28 mgm.), the survival was diminished from 90 to 64 per cent. The odds against this being a chance result are 1300:1.

These low-mineral and low-calcium diets were satisfactory for maintenance and health of uninoculated adult mice over periods of months.

It is concluded that calcium is a limiting factor in the host resistance of Line-A mice to *Salmonella enteritidis* infection.

**STAPHYLOCOCCUS STUDIES. I. TOXIN PRODUCTION.** *E. P. Casman*, Abington Memorial Hospital, Abington, Montgomery County, Pa.

A study of the conditions for the production of staphylococcus toxin was made. It was found that a veal infusion medium containing 2 per cent Difco proteose peptone, 0.7 per cent sodium acetate, 0.5 per cent sodium chloride and 0.3 per cent agar and sterilized in the autoclave was superior to a veal-infusion-free medium containing 2 per cent proteose peptone, 0.7 per cent sodium acetate, 0.5 per cent sodium chloride, 0.1 per cent potassium phosphate (dibasic), 0.1 per cent potas-

sium phosphate (diacid), 0.02 per cent magnesium sulfate, 0.01 per cent calcium chloride and 0.3 per cent agar. Sterilization of veal infusion media by filtration did not result in an increase of toxin production. When the medium was adjusted to pH 6.8, a gaseous atmosphere of 80 per cent oxygen and 20 per cent carbon dioxide and an incubation period from 48 to 60 hours gave the best toxin yields.

Dialysis of a veal infusion medium through cellophane removed most of the substances that could be precipitated by saturation with ammonium sulfate and made possible the preparation of a relatively pure toxin by means of ammonium sulfate precipitation. Yeast extract enriched with dialyzed proteose peptone and 0.7 per cent sodium acetate was equally efficient in the production of a potent toxin that could be purified by precipitation with ammonium sulfate.

**CONCENTRATION OF STAPHYLOCOCCUS TOXOID BY THE KIDNEYS.** *E. P. Casman*, Abington Memorial Hospital, Abington, Montgomery County, Pa.

After intravenous injections of staphylococcus toxoid into rabbits whose sera contained no appreciable staphylococcus antitoxin, heart blood and bladder urine specimens were taken and these titrated for their toxoid contents. The concentration of toxoid in the urine specimens after the toxoid injection was always higher than that in the blood serum samples obtained after injection of the toxoid. The degree to which the toxoid was concentrated by the kidneys varied inversely with the amount of urine excreted. When, for example, 0.015 cc. of urine was excreted per minute, the urine contained 60 times more toxoid per cubic centimeter than did the blood

serum. When 0.35 cc. of urine was excreted per minute, the degree of concentration effected by the kidneys was only 2.5 fold.

DEGENERATIVE CHANGES OF THE NEUTROPHILES IN CLINICAL AND EXPERIMENTAL OBSERVATIONS. *Max M. Strumia*, The Bryn Mawr Hospital, Bryn Mawr, Pa.

Critical study of results of blood examination of 136 cases of lobar pneumonia in adults shows that very valuable data for the prognosis may be obtained. The elements essential to the prognosis in increasing order of importance are absolute number of lymphocytes, monocytes and eosinophiles, and percentage of cells showing cytoplasmic or nuclear degenerative changes. The percentage of young cells (nuclear shift or Schilling's index) is too variable to be of much importance. It may generally be stated that if the percentage of young neutrophiles

is constantly low the case will probably have a favorable outcome but if the percentage of young neutrophiles is high then the outcome may be almost anything.

Lymphopenia, monopenia, eosinopenia and a high number of degenerated cells are very definitely unfavorable signs. It is essential that the following points be thoroughly covered: first, judgment should be based not on a single count but on a series of counts taking into consideration the variations of the various groups of cells from day to day. Second, the preparations must be technically uniform and as good as can be obtained. Third, correlation must be established between the blood picture and the clinical course.

The prognostic value of the blood examination is particularly important because changes in the blood picture usually take place a considerable period of time before a corresponding change in the clinical picture.

# THE SEROLOGICAL CLASSIFICATION OF GONOCOCCI BY COMPARATIVE AGGLUTINATION

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It is only recently that serological methods have attained practical significance in the diagnosis of gonococcus types.

Torrey (1907) and Teague and Torrey (1907) demonstrated differences of type in different strains of gonococci by agglutination as well as by complement fixation and, in addition, by precipitation in rabbit immune sera. They examined 10 strains, of which 6 could be classified into 3 serological groups. In a later investigation, Torrey and Buckell, (1922) by serological examination, endeavored to obtain data concerning the frequency of the individual types in a large number (77) of strains, some of which were twelve years old. Since they encountered great difficulties in the production of suitable diagnostic sera, they obtained unsatisfactory results both by agglutination and absorption. They assumed that the gonococcus antigen is very unstable as they did not find any sharp differences of types in the 3 serological groups (regular, intermediate, irregular) which they finally established. They found several strains whose sera had a very great range of reaction so that the majority of the different strains of gonococci examined were able to absorb several of these 9 sera for the homologous strains. On the other hand, it was not found that all the strains which furnished the overlapping and apparently very good sera, were identical. They were by no means able to absorb all the antisera of those strains which, on their part, had absorbed these "sera with wide antigenic valency."

These authors, therefore, could not combine all the strains which absorbed exactly the same sera, into one group as the



groups would overlap with each other. They finally assigned 39 strains which had absorbed the majority of the 9 sera to the "regular" group, 18 strains which had only partially or totally absorbed a few of the nine sera to the "intermediate" group and 19 strains which had absorbed none of the sera to the "irregular" group. They also observed that with cultivation for a longer period of time an irregular type changed into a regular type. They stated, therefore, that, despite the unstable nature of the gonococcus antigen, there is a general tendency to revert to the regular type. Consequently, they believe that it is not logically permissible to speak of "types" *per se*.

The investigations of several other authors have not led to concordant answers to this question. Direct examination of patients' sera by the commonly used antigens of "wide valency" could not, for understandable reasons, lead to definite conclusions (experiments of M. Stern).

Tulloch (1922) examined only freshly isolated strains from acute and subacute gonorrheal urethritis of males for their types, using *only one* high titer rabbit immune serum and its homologous strain. With this antiserum he selected those strains which, by absorption, decreased the titer of the immune serum for its homologous strain to  $\frac{1}{8}$  or  $\frac{1}{4}$  of its original value. These strains were assigned to type I. He found 43 strains which decreased the titer to less than  $\frac{1}{8}$  and 29 "closely related" strains which decreased the titer to less than  $\frac{1}{4}$  of its original value. The remaining 28 strains did not influence the titer of the type I serum at all or only lowered it to  $\frac{1}{2}$ . At the end of both series, the remaining strains were further classified by absorption, using antisera some of which were obtained from old and some from freshly isolated heterologous strains. Twenty strains could be identified by means of 4 sera, 3 of which certainly belonged to completely different serological groups. Eight strains remained uncertain. Nevertheless, Tulloch classified his type I strains according to the height of titer of agglutination.

Atkin (1925) has objected not only to the investigations of Torrey and Buckell but, in general, to the entire serological classification. This author had already applied cultural methods of differentiation on special media (alkaline pea-broth agar in a

semi-solid state) to meningococci with particular reference to colony morphology. He made a comparison between Gordon's type II meningococcus and a strain of the same author's type I, which had been cultivated for a long period of time, and showed that such a degenerated type I could not be differentiated from a type II on the basis of colony morphology. When incubated for a long period of time on this culture medium, the shape of the freshly isolated and undegenerated type I could be easily distinguished because it formed papillae and exhibited a characteristic form of colony with a halo while type II had neither papillae nor halo.

Atkin found quite analogous varieties of gonococci and has established a parallelism of sharp serological differences between the antisera of both of these colony types. He was able to show, in a comparative study of a great number of chronic and of some acute cases of gonorrhea, that gonococci which had been isolated from chronic cases were acted upon by a type II serum obtained from a 2-year-old cervix strain which exhibited all the characteristics of a degenerated type I or were influenced by the sera of both types. Gonococci which had been isolated from acute cases were agglutinated either by the serum of a strain which formed papilla-bearing colonies (type I) or remained altogether unaffected by either serum.

However, these investigations did not clarify the question of the importance and frequency of the single types of gonococci.

Another method, namely, the cuti-reaction, has played an equally important rôle in the indirect demonstration of gonorrhea by immunological procedures. Because of the sensitivity of healthy individuals, however, this reaction was repeatedly rejected. This sensitivity was a consequence of the use of suspensions of gonococcus cultures or its soluble extracts as test antigens.

In a preceding paper (Casper, 1930) we have demonstrated that in a great number of cases a protein-free derivative which had been obtained from a gonococcus strain designated by us as type I, gave a strictly specific reaction, i.e., normal persons were not at all affected, while gonorrheal individuals, as a rule, showed characteristic skin reactions.

As was emphasized in that paper, this antigen was obtained

from a strictly type-specific gonococcus strain which was not agglutinated by a high-titer heterologous antiserum. The antigen was examined by precipitation with the antisera of both types and was demonstrated to be strictly type-specific. Conversely, the protein-free antigen obtained from the more infrequent type II was also found to be strictly type-specific.

The purpose of the present paper is to report the results of the fundamental diagnostic agglutination tests which preceded the production of these type-specific antigens (Casper, 1937). In these agglutination tests for the recognition of the different types of gonococci we started with the assumption, in accordance with earlier experiments on pneumococci, that strains from fresh cases should be most suitable for the demonstration of type characteristics. Tulloch also followed this principle and laid stress on the fact that he limited himself to only acute and subacute cases of gonorrhea. We have gone even further and have examined, in general, only quite recent, untreated cases. In addition, we attempted, above all, to demonstrate the existence of 2 types which were completely different from each other and which were not overlapping in agglutination. This requirement appeared to be necessary because, in the case of pneumococci Avery and Heidelberger found that carbohydrates of a specific chemical structure represent the type-determining factor and that auto-lyzed type-specific cultures react with the antisera of heterologous types. In the same way a partially degenerated culture might show an overlapping reaction. In such cases, therefore, these types could be differentiated from each other only by an absorption test.

But, in the interpretation of an absorption test, the question always arises as to whether the overlapping reaction was caused by the common portion of the antigen or by the relationship of the type-specific factors. The solution of all these questions is simplified if, as in our experiments, the diagnostic sera are prepared with 2 completely different types which have no sort of relationship between the type-specific factors.

*Culture media:* we have taken into consideration still another difficulty, namely, that cultures on artificial media frequently

tend to agglutinate spontaneously in saline or in normal rabbit serum. In our experiments, we first used 5 per cent horse blood agar and later the blood-water agar (Casper, 1929) on which gonococci showed an optimum growth and did not degenerate so quickly. But even when the best possible culture media are selected the tendency towards spontaneous agglutination cannot always be excluded. Therefore, we never use such strains for the production of antisera and have considered them separately in establishing the frequency of the types.

*Preparation of serum:* in the preparation of our first batch of sera we followed the precautionary measures advised by Tulloch namely, the use of killed vaccines in order to avoid autolysis and the production at one time of all the antigen necessary for the entire process of immunization. Although Torrey and Buckell consider the temperature of 60°C. used by Tulloch as too high and deleterious to the antigen, we obtained good results with this method. But we also obtained good sera with living cultures which had been freshly prepared for each injection if the strains used had not been isolated too long previously. We obtained a good serum from a 1-year-old laboratory culture of our strain 1 (type I). Other strains of this age, however, were not suitable because they overlapped too much.

A partly living, partly dead vaccine was injected intravenously every 4 to 6 days in increasing doses of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1 to 16 loops. After the injection of 8 loops, a trial bleeding was made and agglutination tests performed. After the injection of 16 loops, the animals were exsanguinated and phenol added to the serum.

To find suitable types we proceeded in the following manner: rabbits were first immunized with 3 freshly isolated strains. The sera of these 3 strains proved to be identical in the absorption and had a titer of 1:800 to 1:1600. A number of freshly isolated strains were then examined for agglutination in 1:25 to 1:1600 dilutions of these 3 sera. The sixth and seventh strains examined did not react at all with these sera, not even in the strongest concentration (1:25). A high-titer antiserum was prepared with one of these strains. This antiserum strongly agglutinated the second strain which also did not react with type I serum. The

**TABLE 1**  
*Preparation of the diagnostic sera*

STRAIN AND SERUM NUMBER	PERIOD	GROUP	TYPE	PREPARATION OF THE ANTIGEN	AGE OF CULTURE AT START OF IMMUNIZATION
1	First	1a	I	Organisms killed at 65°C., preserved with phenol	38 days
2	First	1a	I	Organisms killed at 65°C., preserved with phenol	38 days
3	First	1a	I	Organisms killed at 65°C., preserved with phenol	38 days
10	First	1a	I	Organisms killed at 65°C., preserved with phenol	86 days
41	First	1a	I	Organisms killed at 60°C., prepared freshly for each injection	7 days
41	First	1a	I	Living	7 days
82	Second	1b	I	Living	39 days
83	Second	1b	I	Living	35 days
76	Second	1e	I	Organisms killed at 60°C., prepared freshly for each injection	8 days
75	Second	1b	I		8 days
		after absorption			
98	Third	1a	I	Organisms killed at 65°C., preserved with phenol	20 days
7	First	2a	II	Organisms killed at 65°C., preserved with phenol	34 days
25	First	2a	II	Organisms killed at 65°C., preserved with phenol	26 days
84	Second	2a	II	Living	30 days
57	Second	2b	II	Organisms killed at 65°C., preserved with phenol	10 days
72	Second	2d	II	Organisms killed at 60°C., prepared freshly for each injection	8 days
116	Third	2b	II	Living	10 days
81	Second	Irregular	I + II Heter- ologous	Living	3 months
51	Second	3		Organisms killed at 65°C., preserved with phenol	4 days
41a	The same strains as above only after long cultivation		I	Organisms killed at 100°C., prepared freshly for each injection	½ year
41a			I	Living	½ year
1a			I	Organisms killed at 60°C., prepared freshly for each injection	2 years
25a			II		1½ years

type I strains (1 to 3), on the other hand, remained completely (1:25) uninfluenced. From this it was concluded that the type-specific factors of both types were completely different from one another. In a manner similar to that used with pneumococci, therefore, it seemed possible to make a classification of types by means of simple agglutination without the use of the absorption test.

Representative results are shown in table 2. We regarded as definitely type-specific only those strains which were agglutinated

TABLE 2

*Plan for the classification of the specifically agglutinating type I and type II groups and the strains belonging to other subgroups according to their content of co-agglutinins and the titer of the specific agglutination*

GROUP	TITER OF COMPARATIVE AGGLUTINATION IN		CO-AGGLUTINATION	RATIO OF TITER TYPE I:TYPE II
	Type I sera	Type II sera		
1a	800 to 1600+	25- to 50+	None or slight	16:1
b	400+	25- to 50+		8:1
c	800 to 1600+	100+	Relatively strong	8:1
d	1600+	200+		8:1
e	400 to 800+	100 to 200+		4:1
2a	25- to 50+	800 to 1600+	None or slight	1:16
b	25- to 50+	400+		1:8
c	200+	1600+	Relatively strong	1:8
d	100 to 200+	400 to 800+		1:4
3	25-	25-	None	0:0

by 1:400 to 1:1600 dilutions of the corresponding serum but which were not influenced by heterologous serum in dilutions greater than 1:50. If co-agglutination took place up to 1:100 or to 1:200 but a higher titer was reached in the antiserum of the heterologous type we have likewise considered this strain as definitely classified. Differential diagnosis by comparative agglutination can still be made if a titer of 1:400 to 1:800 is followed by a co-agglutination in 1:100 to 1:200 heterologous antiserum. (Strains with the relatively high co-agglutination-agglutination titer ratio 1:4.)

Controls were done in all cases consisting of: (1) normal rabbit serum in various dilutions and (2) one control in saline. We regarded as uncertain all reactions in which a strain was spontaneously agglutinated in saline (even to a slight degree) or influenced by normal rabbit serum. These we have gathered into a special group which will not be taken into consideration in the calculation of the frequency of the types. According to our preliminary tests with strains from chronic gonorrhea it seems that these strains, particularly, tend to agglutinate spontaneously to a lesser or greater degree.

In addition to both of these principal types, we distinguished those types which are not influenced by either serum and which, therefore, probably belong to another type (shown under the heading group 3 in table 2).

Furthermore, strains which were sensitive to the same degree in both sera and strains which were only weakly sensitive in both sera (never more than 1:200) have been found. In the latter case a difference in titer in one serum of 1:200 and 1:50 in the other serum seemed to be insufficient for diagnosis without the aid of the absorption test (cf. table 4).

Since most of the strains could be typed by comparative agglutination, we have used the absorption test in only a small portion. In the absorption test, both sera were simultaneously absorbed for the respective test strain. The difficulties which resulted from this, especially in those cases in which the strains were degenerated as a consequence of long cultivation, will be later discussed in detail.

The confirmation of the diagnosis by preparation of an anti-serum from the examined strain was relatively seldom necessary. However, sera were prepared with 18 strains. Moreover, purified protein-free carbohydrate fractions (Casper, 1937) were also prepared from 6 strains and the type-specificity determined by examination with both antisera. Conversely, some of the antisera were established as type-specific by precipitation tests with both carbohydrate fractions.

In the following we report on the results of our investigations which extended over a period of several years (1927-1933). The

TABLE 3

*Comparison of the type I and type II strains of the first period (1927 to 1930) which had clear controls by means of crossagglutination tests in type I sera (on the left) and type II sera (on the right)*  
 The test material consisted of living gonococci suspended in saline

Strain Nr	IMMUNE SERA 1, 2, 3 (TYPE I)																			IMMUNE SERA 7 (TYPE II)										25 (TYPE II)		7 (TYPE II)							
	1*†	2*†	3*†	10*	14	15	16	17	19	20	26	36	41	42	43	6	7*	25*	1	2	3	10	14	15	16	17	19	20	26	36	41	13	41	42	43	6	7	25	
1:1,600	+	+	+	+	±	±	0	+	0	0	0	0	0	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1: 800	+	+	+	+	±	±	±	±	±	±	±	±	±	±	±	±	±	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1: 400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1: 200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1: 100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1: 50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Controls: homogeneous in saline; negative in 1:50 to 1:400 normal rabbit serum

\* Twenty-one strains could be classified without supplementary tests.

† Seven strains were conclusively classified by production of an antiserum.

‡ Five strains were conclusively classified by absorption test.



reason for this was that we scrupulously selected only acute untreated cases of gonorrhea as the source of our strains.

Table 3 shows the results obtained with 21 gonococcus strains with good controls. These could be immediately classified on the basis of differences in titer when they were tested simultaneously with type I and type II sera. This table also shows that 11 of the 21 strains remained perfectly homogeneous even when treated with the strongest concentration (1:50) of type II serum while they reacted to the maximum titer or at least to a 1:400 dilution of type I serum.

Three other strains (19, 26, 36) gave a + reaction with the heterologous type II serum but in no dilutions higher than 1:50; a fourth strain (41) also gave a + reaction with a 1:50 dilution. Strain 41, however, reacted with a 1:1600 dilution of type I serum. All cases in which the titer is increased more than eight fold belong to our group 1a.

Strain 11 which reacted  $\pm$  with a 1:400 and + with a 1:200 dilution of type I serum and which did not react with type II serum should be classified with our group 1b.

Only with strains 42, 43, and 44 is the titer difference lower and co-agglutination somewhat higher. Strain 43, which has a four times higher titer (+ reaction in 1:400 type I serum and + and  $\pm$  reaction with 1:50 and 1:100, respectively, type II serum) belongs to group 1e. Strain 42, which reacts four times more strongly with type I serum (1:800  $\pm$  and 1:400 +) than with type II serum (1:200  $\pm$  and 1:100 +) also belongs to this group. Strain 44 which gives a + reaction with 1:200 type II serum and a  $\pm$  reaction with 1:1600 type I serum belongs to group 1d.

The last 3 strains examined (6, 7, 25) belong, undeniably, to type II. They all reacted with 1:800 type II serum while they showed a perfectly negative reaction with 1:50 type I serum.

The reading of table 4, which presents the results of the investigations of the second period, is somewhat more difficult because the results are not so clear cut. In it are found those type I and type II strains which can be classified only by absorption as well as those strains which, according to the absorption test, are different from both types. Along with the absorption

TABLE 4

*Comparison of the type I and type II strains of the second period (May 1930 to May 1931) which had clear controls by means of cross agglutination tests in type I sera (left) and type II sera (right)*

Different kinds of antigen suspensions, heated suspensions of some of the strains were examined

Strain Nr	Type I serum 41										Type II serum 57																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
	ANTIGEN HEATED TO 100°C.		ANTIGEN LIVING SUSPENSION		ANTIGEN HEATED TO 56°C.		ANTIGEN LIVING SUB-PENSION		ANTIGEN HEATED TO 100°C.		ANTIGEN LIVING SUB-PENSION		ANTIGEN HEATED TO 56°C.		ANTIGEN LIVING SUB-PENSION		ANTIGEN HEATED TO 56°C.		ANTIGEN LIVING SUB-PENSION																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
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Key to symbols:

\* Absorption tests were made with these strains. This symbol also indicates the type serum with which absorption was successful.

† Strain 51 was classified as a heterologous type.

‡ According to absorption test, these strains (52, 62) were classified as belonging neither to type I nor to type II.

test the indirect method of identification was used, namely: examination of the agglutinins and precipitins produced by the immunization of rabbits with the strains in question.

Moreover, the diagnostic sera I as well as II, have been changed several times.

In the preparation of the antigen we have employed all possible variants.

Of the 3 strains (47, 48, 49) which were heated to 100°C., only strain 49, which reacted four times more strongly in type I serum than in type II serum, could be identified as of type I by the difference in titer.

Strains 47 and 48 could be identified as type II only with the aid of the absorption test. It is remarkable that strain 47 reacted twice as strongly in a high titer II serum (25) and that strain 48 gave an equally low reaction (1:100) in both sera. According to our scheme, these two strains had to be assigned to groups 5d and 6b, respectively (table 5).

Whether it can be concluded from this that the type II antigen is particularly sensitive to heat remains uncertain. The fact that, in testing the thermostability of the isolated type-specific carbohydrate fractions, precipitation with the undiluted type II substance decreased markedly after heating while the substance prepared from a type I strain withstood heating, speaks in favor of this conclusion.

Two (65, 67) of the 7 strains, which were tested with the unfiltered I serum 41, reacted strongly with I serum and  $\frac{1}{2}$  to  $\frac{1}{4}$  as strongly with type II serum (groups 1b and 1c, respectively).

Strain 57 is a distinct type II as it does not react at all with I serum and gives a + reaction in 1:400 II serum. Strain 56 reacted  $\pm$  in 1:200 I serum and + in 1:400,  $\pm$  in 1:800 and  $\pm$  1:1600 II serum.

In the determination of the end titer of the reactions carried out in several concentrations, we decided to count only the first  $\pm$  following a + reaction for the comparison of the titer with that in the heterologous serum. Experience had shown that when these weak reactions were repeated variations in the end titer were frequent.

Strains 51, 52 and 62 in the table occupy a special position since these 3 strains belong neither to type I nor to type II. Strain 51, particularly, showed no reaction (— in 1:25) in either type I or type II serum. The 2 strains, 52 and 62, reacted in 1:100 and 1:400 I serum and 1:100 and 1:200 II serum and, therefore, are approximately the same in both sera. In the absorption test, however, strain 52 was unable to remove the specific agglutinins for either of these 2 types while strain 62, as a special type, removed the agglutinins from *both* sera. Of the 6 strains which were tested by the filtered serum 41f, 3 were heated to 56°C. and 3 were tested while living. Of the former, strains 76 and 78 proved to be type I by comparative agglutination and belonged, respectively, to the groups 1e and 1c. The classification of strains 75 and 76 was confirmed by the production of an immune serum and the testing of its agglutinins. As further confirmation absorption tests were repeated with the immune serum. Strain 75 had been previously identified as a type I by the absorption test. The high co-agglutination of strain 75 and the relatively high co-agglutination of strains 76 and 78 warrants the conclusion that the gonococcus antigen which gives an overlapping reaction is not destroyed by heating to 56°C.

Of the 3 unheated cultures which were tested with serum 41f, strains 82 and 83 gave a + reaction with 1:400, and strain 84 a + reaction with 1:50 I serum. The reactions were reversed in type II serum, strain 84 giving a + reaction with 1:800 and strains 82 and 83 negative reactions with 1:50 so that diagnosis can be clearly made. The classification was confirmed by the preparation of immune sera and the examination of their agglutinins.

The last strain examined (72) showed itself to be a type II as it agglutinated to the end point (1:1600) in type II serum (57) while the I serum (1a) with its overlapping valency agglutinated it only to 1:200, i.e., was  $\frac{1}{8}$  as strong. In this case also the diagnosis was confirmed by the preparation of an immune serum. Of the 17 strains tested, 7 were clearly type I and 3 clearly type II. Two other strains were proven to be type II by the use of the absorption test. The percentage of type I is, therefore, much

smaller in this series than in the first series. To these must be added the 3 strains classified as heterologous and the irregular strains excluded from this table. It seems possible, therefore, that an epidemiologically significant change in the types is taking place.

In consideration of the importance of this problem, we decided, in 1933, to resume these experiments and give the results obtained with 15 strains with good controls. In these experiments, we used the diagnostic sera 82 (type I) and 84 (type II). These sera were prepared from the type-specifically reacting strains just mentioned shortly after their isolation.

In this period again a definite preponderance of type I can be shown. Nine strains reacted strongly with type I serum and only weakly with type II serum. Of these, 2 reacted up to 1:100 in type II serum and, therefore, belong to group 1e.

Three strains reacted with a 1:400 to 1:800 dilution of type II serum and much less strongly with type I serum (1:25, 1:50). They, therefore, belong to groups 2c and 2d respectively. Three strains only reacted with 1:25 dilution of type I and type II serum. They, therefore, are heterologous types of group 3.

Eight strains of this period, belonging to the "irregular" or "degenerated" strains will be discussed later.

Absorption tests were not performed during this period. At this time, again, the majority of the strains could be easily classified by "comparative agglutination." As in the first period, type I was predominant and the absorption test was unnecessary.

If we divide the material thus far considered as to the frequency of types I and II and the heterologous types which can be identified by comparative agglutination, we find, in the total of 53 strains:

	TYPE I	TYPE II	HETEROL- OGOUS	RELATED TYPES I AND II BY ABSORPTION	TOTAL
First period.....	18	3	0	0	21
Second period.....	8	6	2	1	17
Third period.....	9	3	3	0	15
Total.....	35	12	5	1	53
In per cent.....	66%	22.6%	9.4%	1.8%	

If we make our judgment concerning the frequency of the types only on the basis of cases which are quite clear diagnostically, we find a great preponderance of type I in the first as well as in the third periods. Totalling the cases of all 3 periods, we find type I present in 66 per cent, a figure which approaches the result obtained by Tulloch (72 per cent). In the first period the percentage is much greater (85 to 86 per cent); in the second period type II is relatively more frequent (37.5 per cent) and type I more rare (50 per cent). In this and in the third periods, there occur 5 strains which are classified as belonging to "neither type I nor type II." Two of these strains were thus classified by absorption and 2 by the complete absence of any effect by either serum. In addition, 1 strain (62) showed the striking characteristic that it could remove, to a high degree, the agglutinins for both types. We shall return to a detailed discussion of this—as yet not clearly explained—group of strains which seemed to play an important rôle in chronic gonorrhea.

#### *The irregular strains*

These figures do not correspond perfectly with actual conditions. They must be corrected by considering the results of irregular and degenerated strains.

As "irregular strains" we understand those with which we did not obtain a clear result by comparison of the titer in both type sera. With some of these strains, neither serum was effective in dilutions higher than 1:200. In these cases, the objection might be raised that we are dealing only with co-agglutination, whereas, if a heterologous serum had been used a higher titer might have been reached. With other of these strains, on the other hand, both sera were effective in dilutions much higher than 1:200. In all of these cases, the absorption test should have been used. But, just in these cases, the absorption test was unsuccessful. In order to achieve a uniform understanding with later investigators, we therefore tried to form a plan, for the classification, according to the height of the titer in the sera of both types, of those strains whose types could not be determined by comparative agglutination.

The resume shows the frequency and the classification into the different groups for all 3 periods.

In 8 cases we obtained a higher titer in type I serum than in type II serum; in 5 cases the titer was higher in type II serum than in type I serum and in 8 cases the titer was exactly the same in both sera. Strong reactions (above 1:200) occurred eleven times among these and below 1:200 ten times. Of the 11 cases with a high titer, 2 showed a four times stronger agglutination (up to

TABLE 5

*Plan for the classification of the irregular strains which had clear controls but which could not be typed by means of comparative agglutination*

	NUMBER OF IRREGULAR STRAINS	GROUP 4a	GROUP 4b	GROUP 4c	GROUP 4d	GROUP 5a	GROUP 5c	GROUP 5d	GROUP 6a	GROUP 6c
First period re- action in	4	1	1	None	1	None	None	None	1	None
	Serum type I	1:3200±	1:1600		1:200				1:800±	
	Serum type II	1:800±	1:800		1:100±				1:800±	
Second period reaction in	11	1	3	None	None	None	2	1	1	3
	Serum type I	1:3200±	1:3200				1:50	1:100	1:400	1:100
			1:400				1:50			1:100
			1:400							1:100
	Serum type II	1:800+	1:1600				1:200	1:200	1:400	1:100
Third period, reaction in			1:200				1:200			1:100
			1:200							1:100
	6	None	None	1	None	2	None	None	1	2
	Serum type I			1:100		1:400			1:400	1:200
						1:200				1:200
	Serum type II			1:25		1:800			1:400	1:200
						1:400				1:200
Total.....	21	2	4	1	1	2	2	1	3	5
Type I > Type II						Type II > Type I			Type I = Type II	

1:3200) in type I serum than in type II serum. However, we did not feel justified in explaining the high agglutination (1:800) in type II serum as co-agglutination. Furthermore, the great number of strains which react exactly alike in both sera is striking; among these were strains (31, 86 and 119) which had a titer of 1:400 to 1:800 in both sera and 3 to 4 strains (55, 94, 104, 53) with a lesser titer (1:100 to 1:200) in both sera. As a whole, type I also predominates in these 21 cases.

Since in a few random tests absorption did not succeed and shed no light on the problem, it was not performed in these cases.

#### THE DEGENERATED STRAINS

In the group of degenerated strains we assembled all those which did not show perfectly homogeneous controls in saline or in normal rabbit serum. As was previously mentioned, we came to this conclusion by the experience that a reaction in saline or normal rabbit serum must be regarded as a sign of degeneration; and the results showed that such strains also acted very irregularly with immune sera. Such strains are probably also more sensitive in their reactions with the different immune sera which, in different animals, do not always contain equal quantities of normal agglutinins.

Altogether we found 35 of these degenerated strains. In the first period, 8 strains were found to be degenerate.

In a few cases, (strain 18), in which the reaction in type II serum is negative while it reaches 1:1600 in type I serum and  $\pm$  in 1:50 normal serum, perhaps the repetition of the test and titration of the normal serum would have shown that this strain was only transitorily influenced by normal serum. Another strain (27) too, which shows a homogeneous control in normal serum and only a slight  $\pm$  reaction in saline, can be considered to be a type I only if one assumes selective influence by type I serum. However, we have recorded these strains here in order to show the different grades of degeneration.

Table 6 combines the degenerate strains of the second and third periods. In it, however, are presented only those strains which showed differences in titer and which were examined by the absorption test or those similar to strain 77 which gave a  $\pm$  reaction in normal serum and the same reaction in the sera of both types and, therefore, are to be considered as most probably of heterologous type.

The strains examined by absorption (50, 61, 59, 73) are marked by a star. It was found that strain 61 absorbed the sera of both types to a large extent.

Strains 50 and 73 showed clear controls during absorption and



exhausted the serum for their own antigen, an indication that at the time of the absorption test they had recovered from their

TABLE 6  
*Degenerated strains of the second and third periods*

Strain Nr.	SECOND PERIOD, ANTIGEN HEATED TO										THIRD PERIOD, ANTIGEN LIVING	SECOND PERIOD, ANTIGEN HEATED TO										THIRD PERIOD, ANTIGEN LIVING
	100°C.		56°	100°	56°	100°C.		100°C.		56°		100°	56°	100°C.								
	Immune sera type I											Immune sera type II										
	41	76		41	76	41		82		25		57				25			84			
46	69	77	50*	73*	61*	59†	115	101	46	69	77	50*	73*	61*	59†	115	101					
1: 3200	0	0	.	.	+	.	+	0	0	0	+	.	.	0	.	+	0	0				
1: 1600	+	0	±	+	+	+	+	0	0	0	+	±	+	0	0	+	0	0				
1: 800	+	0	±	+	+	+	+	0	0	0	+	±	+	+	0	+	0	0				
1: 400	+	0	±	+	+	+	+	0	0	0	+	±	+	+	+	+	+	+				
1: 200	+	+	±	+	+	+	+	0	0	+	+	±	+	+	+	+	+	+				
1: 100	+	+	±	+	+	+	+	0	0	+	+	±	+	+	+	+	+	+				
1: 50	+	+	±	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+				
1: 25	+	+	±	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+				
Control: Nor- mal serum																						
1: 400	0	0	±	0	0	+	+	0	0	0	0	±	0	0	+	+	0	0				
1: 200	0	0	±	0	0	+	+	0	0	0	0	±	0	0	+	+	0	0				
1: 100	0	0	±	+	+	+	+	0	0	0	0	±	+	+	+	+	0	0				
1: 50	0	+	±	+	+	+	+	±	±	0	+	±	+	+	+	+	±	±				
1: 25	+	+	±	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+				
Saline control	0	0	±	0	0	+	+	0	0	0	0	±	0	0	+	+	0	0				

Key to symbols:

\* Absorption test showed that: Strains 50 and 73 were "neither type I nor type II" = heterologous. Strain 61 was "type I and type II." The absorption test was unsuccessful with strain 59.

† 9 strains which, similar to strain 59, were agglutinated to the end titer by the sera of both types must be added to the second period. Six of these were agglutinated by 1:400 normal serum and 3 by 1:100 normal serum. Living suspensions of the latter were used as antigen. Nine strains which reacted like strain 59 must be added to the third period.

. Not done.

degeneration. However, they were unable to influence the I and II agglutinins; they therefore had to be assigned to the heterologous types, with this result considered as not final.

Strain 59 could not even remove its own agglutinins, as one would expect from the experimental conditions applied. Strain 61 absorbed the specific agglutinins from the sera of both types to a high degree so that it is related to both of them.

In the second period, strain 46 could be assigned to type I and strain 69 to type II on the basis of titer difference alone. In the third period, similarly, strains 115 and 101 could be classified as type II.

Of the remaining strains not specified in this table, 9, which reacted like strain 59, must be added for the second period. Of these, 6 strains were agglutinated in dilutions of normal serum up to 1:400 and 3 up to 1:100. Nine strains of the third period agglutinated up to the end titer in both immune sera as well as in normal serum.

In 23 of these 35 cases no indirect methods were applied. In 1 case the absorption test was unsuccessful and in the other 11 a diagnosis could be made only with reservations.

If we now take into account, in the classification of types, the results obtained in the examination of the irregular and degenerated strains, we get the following picture:

	53 STRAINS, GREATEST NUMBER OF DIAGNOSED TYPES. STRAINS WITH GOOD CONTROLS		74 STRAINS, WITH ADDITION OF THE 21 IRREGULAR STRAINS	109 STRAINS, SMALLEST NUMBER WITH ADDITION OF THE 35 DEGENERATED STRAINS	
		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>
Type I . . . . .	35	66	47 3	4	35 7
Type II.....	12	22 6	16 2	3	13 7
Heterologous.....	5	9 4	6 7	3	7 4
Type I and II . . . . .	1	1 6	1 3	1	3 6
Not classified.....	0		28 3	24	39 4
Altogether a diagnosis was made in per cent . . . . .		100	71 5		60 6

From this summary it is obvious that the number of type I strains, after addition of the degenerated and undiagnosed irregular strains omitted in the first summary, is relatively much smaller but, nevertheless, constitutes almost half of all the strains. Type II is the most frequent of the other types.

Altogether we find 71.1 per cent of the cases diagnosed. In this number about 10 per cent (11) of the degenerated strains, in which the diagnosis was made with reservations, are *not* included.

We consider this a favorable result for the evaluation of our methods; for, as we mentioned above, no definite conclusions as to the degenerated strains and, as will be shown later, as to most of the cases, can be drawn from the use of the absorption test. Therefore, according to our experiences with strain 59, it cannot be expected that the results of absorption tests with the 21 irregular strains would have essentially decreased the percentage of type I.

Even 47.3 per cent would mean a great spread of the strains which we have designated as type I. Of course, it seems important to us that 16.2 per cent belong to type II. 6.7 per cent of the cases, we designate as heterologous, are also epidemiologically important. The percentage, like that of type II, varied in the different periods.

On the other hand, the type designated by us as "related to types I and II" seems to have a lesser significance in acute cases of gonorrhea, although among the irregular strains there were 3 which reacted equally high in both sera and 5 which reacted to a lesser degree in both sera and which, perhaps, should be assigned to this type. This type seems to be of rather great significance in the strains isolated from cases of chronic gonorrhea.

#### THE STRAINS FROM CHRONIC GONORRHEA

We have examined only a few strains from chronic gonorrhea (6 in the first period and 10 in the second); all these strains had a certain tendency towards agglutination in normal serum; 2 strains were also agglutinated in saline. The majority of them, therefore, have been classified with the degenerated strains.

It was found that all these strains were highly affected by the sera of both types. This could also be proven by the absorption test which was performed with 5 of these strains (34, 37, 38, predominant strain (Tulloch) and 81 which showed clear con-

trols.) They all absorbed the I and II agglutinins to the same extent.

In regard to the frequency of occurrence, this behavior is characteristic of the chronic strains. It is true that this behavior is in contrast to that of pneumococci. No parallel can be drawn with this organism, even when it has lost its specificity. However, the possibility of the relationship of 2 type-specific substances, different from each other, to a third, should not be regarded as outside the realm of feasibility. It must be mentioned that Griffith made a similar observation with streptococci. He

TABLE 7

*Crossagglutination tests in their reciprocal sera, of 4 strains of gonococci (82, 83 (type I), 84 (type II) and 81)*

GONOCOCCUS STRAIN	SERUM 81						SERUM 82					
	1 50	100	200	400	800	1600	1 50	100	200	400	800	1600
81	+	+	+++	++	++	+	(+)	(+)	(+)	0	0	0
82	(+)	(+)	(+)	+	(+)	±	++	++	+	+	+	±
83	+	++	++	+	+	+	+	+	+	+	(+)	(+)
84	(+)	+	++	++	+	(+)	+	0	0	0	0	0
	SERUM 83						SERUM 84					
	1 50	100	200	400	800	1600	1.50	100	200	400	800	1600
81	+	+	+	(+)	0	0	+	+	+	+	0	0
82	±	±	(+)	(+)	0	0	+	0	0	0	0	0
83	++	++	++	++	+	(+)	+	(+)	0	0	0	0
84	±	±	0	0	0	0	++	++	++	++	+	(+)

found that one of his types could absorb the agglutinins of 2 heterologous type sera which were strictly different from each other. We prepared an immune serum with strain 81 and examined, with this serum, two I strains (82, 83) and one II strain (84).

As is seen from table 7, serum 81 influenced all 4 strains up to the end titer. Strains 82, 83 and 84, however, proved to be strictly type-specific when examined with the homologous sera (82, 83 (type II)). From this, it seems that there exists a type "I + II."

Strain 81 was also used several times for absorption tests. With only one exception, it absorbed, to the same degree, the agglutinins for most of the type I and type II test strains from their respective sera.

This strain, therefore, offers an analogy to the observations made by Griffith on streptococci. Furthermore, the relationship between types I and III meningococci should be remembered. In his cases, Griffith assumed "phases" similar to those of the paratyphoid bacilli. Here we might refer to an earlier paper by Casper (1928) who, in trying to prepare the carbohydrates of paratyphoid B and Breslau bacilli (in which we worked with purely specific and non-specific phases) had found that the carbohydrates obtained from all four variants were apparently identical. They were all heavily precipitated by specific Breslau serum while they were not at all or only very little influenced by non-specific Breslau serum or specific Schottmueller serum. From this, it follows that the "serological factor" is contained in all these phases of the paratyphoid bacillus but "is masked" in the production of antibodies, in the rabbit, by the specific Schottmueller and the non-specific Breslau bacillus. Another serological factor must, therefore, be dominant in these 2 phases. The conception is derived, therefore, that during a change of phase the species-specific carbohydrate can become dominant for the non-specific type of Schottmueller, while the same carbohydrate, which represents the dominant haptene of the specific Breslau type, is masked in the specific type of Schottmueller.

If one assumes, therefore, that in the cultivation of gonococci a change of phase eradicated the serological differences between the types, in a manner similar to that of the paratyphoid bacilli, the explanation of the following is made clear: (1) the relationship between strain 81 and types I and II, and (2) the frequency of the appearance of such mixed phases, (not only in chronic cases of gonorrhea but also in some acute cases) in which equally high agglutination titers were obtained in both type-specific sera, an occurrence which is not infrequent with paratyphoid bacilli.

At that time we thought of this possibility and examined different sera of type I and II gonococci, prepared partly with living

and partly with heated cultures. This was done by means of slide agglutination using several strains of each type in order to demonstrate the combination of the different type-specific strains from two different phases.

The result was not quite uniform. However, a dominant overlapping of the I serum, 82, which influenced a large number of single colonies of almost all type II strains, was always observed. The II serum, 57, on the other hand, acted upon a large number of I colonies. This experiment, which suggests further illuminating observations in this direction, will be discussed in a separate paper.<sup>1</sup>

As was mentioned at the start, we separated the chronic strains, which showed only a relatively overlapping reaction in normal serum, from those obtained from cases of acute gonorrhea. We also tried to explain the overlapping reaction in both type-specific sera as a sign of degeneration.

On this fact is based the conception that normal agglutinins are increased during immunization. Absorption tests have shown, however, that the chronic strains were able to remove the specific agglutinins for the homologous strain from sera of both types. Now, however, our homologous test strains, in fixation experiments, proved to be strictly specific on every occasion, so that it is doubtful whether we can assume that they are acted upon by normal agglutinins. Perhaps one might rather assume that the chronic strains contain both phases and that they are thus also able to remove the agglutinins for the heterologous type.

#### THE "PREDOMINANT" STRAIN OF TULLOCH

For the purpose of comparison, we included this strain here although it had been cultivated for a long time on artificial media. It was kindly placed at our disposal by Dr. A. Cohn (1925). He found it to be equivalent in the complement fixation test to the strain he formerly used.

It seems noteworthy that the predominant strain of Tulloch showed a close relationship to both our types, not only in agglutination but also in absorption. It absorbed the specific agglu-

<sup>1</sup> The Cultural and Serological Behavior of Degenerated Gonococci.

tinins of both the I and II strains and, therefore, proved to be a "type I and II."

It is interesting now to recall how Tulloch selected the type which he later designated as "predominant."

He examined 13 strains in his preliminary experiment. Six of these were used for the preparation of sera. These sera proved to be little different when tested by all 13 strains. By the absorption test, however, 2 types were found, one of which, the so-called type I, was later used for the examination of all of his 100 cases.

Type II and the other heterologous types, however, were not tested at the same time but were only later identified by their corresponding sera. By this method of examination, the differences of titer which he would have obtained by a simultaneous test with a heterologous serum could not become manifest. Certain differences in titer were present in the 13 strains. For, several I strains reacted more strongly and others less strongly with type II serum. Likewise, one I serum exerted a stronger influence on the II strains than did the others. The strain which yielded the most overlapping serum was chosen by Tulloch for his absorption experiments. This strain, therefore, would correspond to our strain 81, i.e., a strain which, until the problem of phase has been clarified, we wish to designate provisionally as "I and II."

#### DISCUSSION

In the course of several years we performed serological tests on 109 strains of gonococci isolated from cases of acute gonorrhea and 16 strains isolated from cases of chronic gonorrhea. All strains were simultaneously subjected to comparative agglutination tests with 2 type-specific sera. A large proportion of the strains showed sharp differences in titer, i.e., they were strongly agglutinated by the serum of one type while the serum of the other type agglutinated them weakly or not at all. The types found were called type I and type II, respectively.

An additional small group of strains impressed us as of heterologous type. These strains were not agglutinated by either of

the type-specific sera, or, if agglutination did occur, the titer never rose over 1:50. From this we must draw the conclusion that there is another type which, however, is seldom found.

These investigations were originally undertaken with the purpose of finding several different type-specific strains of gonococci, isolating the type-specific portion of their antigen and examining their carbohydrate content. We were able to demonstrate that each of our 2 types contained different polysaccharide-like substances to which, as is the case with pneumococci, the specific reaction had to be ascribed.

With the finding of these 2 characteristic types, difficulties arose in our investigations. The diagnostic sera had to be prepared by the immunization of rabbits with freshly isolated strains. All of these sera did not prove to be equally selective when tested with fresh gonococcus cultures. We encountered great obstacles, especially in the second period of our investigations, because of overlapping and weaker reactions. In this period, old instead of freshly isolated strains were used for the preparation of antisera. Thus it was shown that old laboratory strains suffer a change in their antigenic properties. A series of experiments with one group of freshly isolated strains demonstrated, however, that the structure of the whole bacterial antigen is more complicated than appeared to be the case after the first period of experimentation. In order to show the difficulties of the serological differentiation of types, therefore, we have considered each strain as an individual, and have shown its reaction according to various methods of examination.

In the second period we used the absorption test more often for the purpose of classification. In only a few of the cases, however, in which comparative agglutination did not succeed in classifying our strains was any conclusion reached by the use of the absorption test.

The irregular and degenerated strains make up the major portion of the unclassifiable strains. The strains obtained from cases of chronic gonorrhea belong to the degenerated strains. While gonococci of both types I and II (similar to pneumococcus I and II) were strictly specific in agglutination, as were their carbo-



hydrates in precipitation, the cultures obtained from cases of chronic gonorrhea were able to absorb the sera of both types. In only one case of acute gonorrhea with good controls did we find that the specific agglutinins of both sera were absorbed to any great degree (strain 62). It seems, therefore, that these strains with antigenic valencies which overlap both type I and type II only seldom occur in acute gonorrhea while they appear more often in chronic gonorrhea and disrupt the classification. One might wish to classify a separate, "type I + type II." It is uncertain as to whether one must assume that there is a degenerated or special type which is particularly frequent in chronic gonorrhea or which might even be the etiological agent in the chronic disease.

Our bivalent strain (81) according to our experimental results, apparently corresponds to Tulloch's "predominant strain." We cannot judge whether the appearance of degeneration, with which we are dealing here, can be ascribed to the age of the culture and to its continued transplantations on artificial media. We do consider the possibility that a change of phase occurred and refer to similar observations by Griffith on streptococci.

In any event, the results of our investigations show that one must reckon with the appearance of degeneration even with some freshly isolated strains of gonococci and that, in the choice of test strains for the production of immune sera this must be considered.

Under the given conditions, therefore, the technique is not ideal since degeneration, even at the time of isolation cannot be avoided. With further improvement of culture media a greater number of strains will probably be classifiable by means of comparative agglutination.

Despite this, in 71 per cent of the cases we were able to determine the type of the strain by comparative agglutination with our type-specific immune sera. Type I was preponderant among our strains. According to our examinations, however, our type I is not identical with the "predominant" strain which Tulloch found in the majority (72 per cent) of cases.

## CONCLUSIONS

(1) By means of comparative agglutination two different types of gonococci (type I and type II) were found. With the aid of the immune sera prepared with them, we endeavoured to classify a large number of gonococcus strains into either of these types.

(2) The difficulties arising from this procedure are described for each individual strain.

(3) The reproduction of type-specific carbohydrate is indispensable for the solution of the problem of classification.

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# BETA ALANINE AS A GROWTH ACCESSORY FOR THE DIPHTHERIA BACILLUS

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In a series of studies carried out in this laboratory (Mueller, 1935, and Mueller and Kapnick, 1935), it has been shown that various strains of diphtheria bacilli grow heavily upon media containing only amino acids in place of peptone, and a considerably purified liver extract in place of meat infusion. Suitable inorganic salts and a source of energy such as glycerol or lactic acid must be added. More recently, it has been possible to identify two of the constituents of the liver extract essential for the growth of our test strain of the diphtheria bacillus, as pimelic acid (Mueller, 1937a) and nicotinic acid (Mueller, 1937b), acting in connection with one or more other unidentified substances. The higher boiling fraction of a vacuum distillate obtained by both esterification and acetylation of the liver concentrate contained the unknown material. The method of preparing this has already been described in the paper on nicotinic acid.

In an attempt to separate the active substance in pure form, the "acetylated esters" containing it were submitted to careful fractionation. The amount available was but 3.0 cc., representing 300 kgm. of fresh liver. The same distilling apparatus (Rittenberg) used in the earlier work was again employed. In a vacuum of about 0.04 mm. Hg, the active material passed over between about 63° and 78°, but it proved impossible to obtain any marked degree of concentration into any one fraction. With the exception of the lowest, measuring 0.15 cc., which contained relatively less activity, the distillates were recombined. Approximately 1.0 mgm. of this oil, after acid hydrolysis, added to a

suitable control gave the maximal effect in producing growth with the test organism.

In an attempt to obtain a crystalline acetyl compound from the oil, a portion was partially hydrolyzed by short boiling with dilute  $\text{Ba}(\text{OH})_2$  solution. This method was shown by Cherbuliez, Plattner and Ariel (1930) to split the ester linkage of the acetylated esters of amino acids, leaving the acetyl group in place. In this way, and after removing Ba with  $\text{H}_2\text{SO}_4$ , there was obtained a strongly acid material which showed no tendency to crystallize either as the Ba salt, or as the free acid. The development of the acid reaction, however, indicated that the original, neutral oil probably contained esterified  $\text{COOH}$  groups.

All of the remaining active distillate, 1.9 grams, was now more thoroughly hydrolyzed by refluxing with about 200 cc. of  $\text{N}/1$   $\text{H}_2\text{SO}_4$  for six hours. The reagent was accurately removed with  $\text{Ba}(\text{OH})_2$  and the solution, which was neutral, was evaporated to dryness in vacuo. The residue was partly crystalline and partly syrup. Solution in hot ethyl alcohol resulted in obtaining a small crop of crystals, which, however, were without activity, while the alcoholic solution showed maximal activity with about 0.5 mgm. of dissolved material, which was largely oily, but contained some crystals.

The fact that complete hydrolysis yielded a neutral substance, whereas an acid was formed by splitting off only the ester group, indicated that acetylated  $\text{NH}_2$  groups were probably present in amounts equivalent to the  $\text{COOH}$  groups. In other words, the mixture behaved as though composed of amino acids. Since acid-hydrolyzed casein was a component of the control medium, and tryptophane was not required by the test strain employed, none of the usual amino acids of protein could be concerned, and  $\beta$ -alanine suggested itself as a possible constituent of the mixture, the more so, since the work of Williams and Rohrman (1936) indicates that this substance is a part of the "bios" complex.

A series of tests carried out with synthetic  $\beta$ -alanine quickly showed that it possessed in marked degree the growth-stimulating properties of the distillate fraction; a quantity of approximately 10 $\gamma$  in 10 cc. of medium producing its maximal effect, as did about

0.5 mgm. of the residue left after hydrolysis of the distillate. Assuming the active substance of the latter, therefore, to be actually  $\beta$ -alanine, it would be present to the extent of only 2 per cent in the oily material,—a total amount of perhaps 20 mgm. in the  $\pm 1.0$  gram of impure material.

Since, as far as we are aware, no compound of  $\beta$ -alanine has been described which would be suitable for its isolation from such a mixture on so small a scale, no further attempt has been made to determine whether or not it actually is the substance present which is essential for growth. That such is probably the case is indicated by the close parallelism of action, by the fact  $\beta$ -alanine is known to occur in tissue extractive, both free and com-

TABLE 1

COMPOSITION OF MEDIUM	BACTERIAL N
	<i>mgm.</i>
Control alone . . . . .	0 32
Control + $\beta$ -alanine 0 5 . . . . .	0 30
Control + $\beta$ -alanine 1 0 . . . . .	0 36
Control + $\beta$ -alanine 2 5 . . . . .	1 18
Control + $\beta$ -alanine 5 0 . . . . .	1 58
Control + $\beta$ -alanine 10 0 . . . . .	2 38
Control + $\beta$ -alanine 25.0 . . . . .	2 56
Control + $\beta$ -alanine 50 0 . . . . .	2.58

bined with histidine in the form of carnosine, and that the boiling point of the distillate is within the probable range for that of the acetylated ethyl ester of  $\beta$ -alanine. In any case, this compound is so readily available<sup>1</sup> that even were a more complex substance shown to be equally active, the former would probably still be the more convenient.

Carnosine itself is effective in replacing  $\beta$ -alanine, but a considerably greater concentration is required, indicating perhaps that it must be hydrolyzed by the organisms in order to render its  $\beta$ -alanine available, and that this reaction is not readily brought about by the bacteria.

<sup>1</sup>  $\beta$ -alanine may be purchased from the Department of Organic Chemistry, University of Illinois, Urbana.

Table 1 illustrates the effect of  $\beta$ -alanine when added in varying quantities to the control medium. The test strain is the "Allen," used in much of the earlier work. The control has the following composition per 10 cc. of medium, and the methods of inoculation, incubation and determination of relative growth by means of nitrogen are the same as previously described.

*Control*

Casein—HCl hydrolysate . . . . .	0.1 gram
Cystine . . . . .	0.001 gram
Glutamic acid . . . . .	0.050 gram
Lactic acid (as Na salt) . . . . .	0.1 cc.
Salt mixture:	
NaCl . . . . .	0.050 gram
Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O . . . . .	0.025 gram
KH <sub>2</sub> PO <sub>4</sub> . . . . .	0.0035 gram
MgCl <sub>2</sub> ·6H <sub>2</sub> O . . . . .	0.003 gram
Pimelic acid . . . . .	1.0 $\gamma$
Nicotinic acid . . . . .	10.0 $\gamma$

TABLE 2

COMPOSITION OF MEDIUM		BACTERIAL N
		<i>mgm.</i>
Control + carnosine 5.0 . . . . .		0.29
Control + carnosine 10.0 . . . . .		0.21
Control + carnosine 25.0 . . . . .		0.66
Control + carnosine 50.0 . . . . .		0.90
Control + carnosine 100.0 . . . . .		1.13
Control + carnosine 250.0 . . . . .		2.68

A similar experiment using synthetic l-carnosine, for which the writer is indebted to Professor Vincent du Vigneaud of George Washington University, is presented in table 2.

That the effect of  $\beta$ -alanine, together with nicotinic acid and pimelic acid, in replacing meat or liver extract is not unique on the "Allen" strain, is shown by an experiment with four cultures of the Park-Williams No. 8 diphtheria organism. Earlier work (largely unpublished) with organisms of this "strain" from various sources have brought out differences in nutritional requirements involving both amino acids and energy sources. It is therefore

not remarkable to find differences in respect to these three extractive substances. It is evident that  $\beta$ -alanine and nicotinic acid must be present together to obtain appreciable growth of any strain, whereas pimelic acid increases the growth in some instances but not in others.

The results are summarized in table 3.

TABLE 3

COMPOSITION OF MEDIUM	BACTERIAL N			
	A	B	C	D
	mgm.	mgm.	mgm.	mgm.
Control* alone	0 07	0 06	0 10	0.10
Control + pimelic acid 1 $\gamma$ (1)	lost	0 04	0 14	0.10
Control + nicotinic acid 10 $\gamma$ (2)	0 02	0.04	0.18	0.14
Control + $\beta$ -alanine 10 $\gamma$ (3)	0.10	0 06	0.10	0.12
Control + (1) + (2)	0.08	0.00	0.17	0.14
Control + (1) + (3)	0 08	0 07	0 11	0.12
Control + (2) + (3)	0.66	1 99	1 92	2.02
Control + (1) + (2) + (3)	2.32	1.86	2 16	1.84

\* To the usual Allen control 0.001 gram of l-tryptophane and 0.05 cc. ethyl alcohol are added for the Park 8 strains.

Strain A, National Institute of Health, Washington, D. C.

Strain B, Alabama State Health Department

Strain C, New York State Department of Health, strain 5

Strain D, Toronto, Canada.

#### DISCUSSION

It is now possible for the first time to obtain heavy growth of certain strains of diphtheria bacilli on a medium which approaches the term "synthetic." It is true that our present control contains hydrolyzed casein but that this may be replaced by known amino acids is indicated by many experiments not presented here. There is still a deficiency of one or more substances, since our present maximum growth with the "Allen" strain of 2.5 to 2.9 mgm. nitrogen can be readily increased to 3.5 to 4.0 mgm. by the addition of whole tissue extract and certain of its fractions. It does not appear to be worth while to complete the substitution of casein hydrolysate with known amino acids until these deficiencies are better understood, and work is being continued along this line.



It is reasonable to hope, however, that even with this amount of growth, which is as good or better than that obtained on the usual peptone-infusion broth, the metabolism of the organism may be nearly enough normal so that toxin of a reasonable degree of potency will be produced. Experiments in this direction are now being made in collaboration with Pappenheimer, whose recent work (1936) on diphtheria toxin places him in an unusually favorable position to carry on this phase of the study.

#### CONCLUSIONS

1.  $\beta$ -alanine has been found to be a further growth accessory substance for the diphtheria bacillus.

2. This substance, in a concentration of about 1 $\gamma$  per cubic centimeter, together with nicotinic acid in the same concentration and (for some strains) pimelic acid in an even smaller amount, permits the growth of several strains of the organism to the extent of about two-thirds the quantity maximally obtainable with whole tissue extract. It is perhaps not without interest that the cost of sufficient of these substances to prepare 1000 liters of broth would be less than one dollar.

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# VARIABILITY IN MORPHOLOGICAL AND BIOCHEMICAL PROPERTIES OF *CLOSTRIDIUM HISTOLYTICUM* (WEINBERG AND SEGUIN)

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## INTRODUCTION

Weinberg and Seguin reported, in 1916, the isolation of a newly-discovered species of anaerobic spore-bearing bacillus from several cases of gas-gangrene infected war wounds.

A pure culture of this micro-organism, when injected into the muscles of common laboratory animals such as guinea pigs, mice or rats, was found to cause a considerable lysis and liquefaction of the muscle around the injected area in less than 24 hours, the lysis usually continuing until the death of the animal. This organism, because of its particularly pronounced ability to attack living tissue, a property possessed by no other sporulating anaerobes, was named "*Bacillus histolyticus*" by its discoverers.

Although the first strains were obtained from war wounds only, in later years, several investigators succeeded in isolating other strains from many other sources such as soil, sewage and the faeces of healthy persons, thus showing that the organism is much more widely distributed than was originally supposed.

In its biochemical and morphological properties, *Clostridium histolyticum* is closely related to *Clostridium sporogenes*, the most common representative of this genus. Both are strongly proteolytic: when inoculated in milk, a rapid digestion occurs, the medium becoming translucent and finally quite clear; gelatin is rapidly liquefied, usually within 24 hours; abundant growth occurs in most media rich in proteins, whether or not sugars are present.

While the majority of investigators agree upon the carbohydrate fermentation by *C. sporogenes*, much undesirable confusion exists in the literature regarding the fermentation ability of *C. histolyticum*.

The original paper of Weinberg and Seguin (1916) gives only a limited number of the biochemical properties of the new species with no data on sugar fermentation, except the statement that no gas is formed in agar-shake cultures containing sugars. Henry (1917), investigating one of the original strains isolated by Weinberg, reported this strain able to ferment glucose, laevulose and maltose from among the sugars tested.

Results similar to those of Henry were reported by McIntosh and Fildes (1917) for other strains: fermentation was weak, but after 7 days' incubation was distinct with glucose, maltose and starch. Later, the same investigators (1919) reported the fermentation of glucose, laevulose and maltose. It should be noted that if these observations are correct this behaviour towards carbohydrates is the same as that shown by *C. sporogenes*.

Hall (1922), and Reddish and Rettger (1924), found that *C. histolyticum* fermented the same sugars as did *C. sporogenes*. A year later, however, Hall (1923) reported that *C. histolyticum* failed to ferment sugars. Since a reasonable explanation for these anomalies was not evident, the possibility of an unknown contaminant in the culture of the earlier experiments was suggested.

Kendall, Day and Walker (1922), Kahn (1924) and also Torrey (1925) deny the sugar fermenting ability of *C. histolyticum*.

Thus, it is seen that highly contradictory results have been reported by the investigators studying this micro-organism. That this contradiction still persists is evident from the fact that Bergey in the 1934 edition of his "Manual of Determinative Bacteriology" still arranges *C. histolyticum* under the sugar-fermenting anaerobes, whereas Spray (1936) in his "Tentative Key to the Sporulating Anaerobes" lists the same bacteria among the nonsaccharolytic representatives.

Since sugar fermentation is usually considered as one of the important criteria for the identification and classification of the

sporulating anaerobes, the deplorability of such an anomaly need hardly be stressed.

The writer's attention was drawn to the contradictory data as regards fermentation while working on the digestion of tumor tissue by representatives of this group. It was found that one of the seven available strains of *C. histolyticum*, a strain obtained from the American Type Culture Collection and tested immediately upon receipt, fermented glucose with the formation of acid and gas, whereas, all other strains gave negative results. For the special study of tumor digestion, all of the strains were carried on a stock culture medium containing a sterilized transplantable rat tumor tissue suspension (1:5) in a solution of 0.1 per cent  $K_2HPO_4$ , 0.05 per cent  $MgSO_4$  and 0.01 per cent  $FeSO_4$  in tap water.

During a period of four months following the receipt of the several strains of this organism, each strain was transplanted several times on the special tumor tissue medium. The sugar-fermenting ability was then tested again, and it was found that all of the strains gave faint but decidedly positive results. Since great care had been taken to avoid contamination, by testing each tube thoroughly before transplanting into it, it appeared worth while to investigate this phenomenon more closely. As a matter of fact there are two possibilities, which can cause the cultures of *C. histolyticum* to ferment carbohydrates, although they were decidedly negative when received. Though transfers had been made very carefully, nevertheless contamination with a sugar-fermenting anaerobe (fermentation persists after pasteurization) is possible and has to be considered. On the other hand the possibility that we are dealing with a form of variation in which sugar fermenting variants of *C. histolyticum* arise from the ordinary non-sugar-fermenting strains need not be rejected at once.

Bacteriologists have long since been reconciled to the idea that there occur variations in single species of micro-organisms, in biochemical behaviour as well as in morphology and colony form. In this regard we may refer to the classic example of Massini's *Bacillus coli-mutabile*, a bacterium which failed to ferment lactose, but the colonies of which upon continued incuba-

tion, formed small papillae composed of bacteria which are able to ferment lactose, a property which could be transmitted by subculturing from these papillae on new plates, and in which also the new character appeared to be permanent.

That this behaviour of *B. coli-mutabile* is by no means a bacteriological curiosity was proved when, a few years later, more examples could be added. So, variants of *Eberthella typhosa* are described which are able to ferment dulcitol or rhamnose and which were obtained from strains unable to ferment these substrates (Penfold 1910, Müller 1911); *Salmonella paratyphi* behaves similarly towards raffinose (Müller 1911). Variants are also known of *S. paratyphi* which are unable to produce any gas during the fermentation of certain carbohydrates; under certain conditions, however, this lost property can be regained and the variant may be transformed into a true *S. paratyphi* (Pot and Tasman 1932). From cultures of *Corynebacterium diphtheriae* and also from *Shigella paradysenteriae* (Sonne), variants are obtained which are unable to ferment certain carbohydrates and other modifications which strongly ferment these sugars (Goodman 1908, Hobby 1935, Chinn 1936, Sears and Schoolnik 1936).

Though this summary certainly is incomplete it may show that variability in sugar fermentation, especially among the pathogenic micro-organisms, is not a rare phenomenon at all, and that therefore the possibility of a spontaneous variation, leading to sugar-fermenting variants need not be excluded.

It has long since been proved that variation in colony form (rough-smooth variation) also occurs frequently in the group of anaerobic spore-bearing micro-organisms, and special attention has been paid in this regard to *Clostridium welchii* and *Clostridium tetani* (Buchaly 1930, Condrea 1930, Orr *et al.* 1933, Stevens 1935). In agreement with what has been found for most of the other bacteria this rough-smooth variation usually does not involve any change in biochemical behaviour towards sugars.

It is generally accepted that there is no direct correlation between variation in colonial type and variation in metabolism. That such a correlation sometimes occurs however has been shown by Colef (1935) for *Clostridium oedematiens*, a bacterium

belonging to the same group as *Clostridium histolyticum*. This investigator succeeded in obtaining colonial variants from characteristic hairy colonies of *C. oedematiens*; these variants had a pronounced tendency to become smooth, and they were non-pathogenic. Contrary to the typical strains of *C. oedematiens*, however, these variants were able to ferment glycerol.

The results of the present study show a similar, though still further-reaching phenomenon with cultures of *C. histolyticum*; it is shown that fermentation of sugars need not necessarily be ascribed to contamination of the *C. histolyticum* cultures but that it can be due to a similar variability, which makes itself manifest not only in a profound change in biochemical properties of the organism, but also simultaneously in the morphology of the colony.

#### BACTERIOLOGICAL INVESTIGATION

It seemed advisable, first, to make a comparative study of transplants from strains which had been found to ferment sugars and the original non-sugar-fermenting strains, using the tumor-tissue suspension medium.

Peptone agar plates, streaked from a tube culture of each strain incubated for two days at 37°C. in anaerobic jars, showed only minor differences in colony form, which did not justify the conclusion that either contamination or variation had occurred. All of the colonies were practically round and small, and were transparent to opaque.

A different result was obtained, however, when the smears were made on liver-veal agar, a medium advocated by Spray for the cultivation of anaerobes. The growth on this medium was abundant, even after only 24 hours. A marked difference was now evident between the plates obtained from the original pure cultures, and their transplants on tumor tissue suspension. The original pure cultures all gave perfectly round colonies resembling those of streptococci, 1 to 2 mm. in diameter, opaque, and with a decolorized zone around each colony on the dark brown culture medium. The transplants on tumor-tissue suspension showed, for the greater part, similar colonies. However, a small portion

of the colonies had irregular margins, some even having short thick shoots protruding from the periphery. With a few colonies, this was so pronounced that the agar around the colony was covered with a network of filaments.

Of importance is the fact that between these two extremes, the regular round and the threadlike colonies, all kinds of intermediate stages were observed. As all the plates had many absolutely isolated colonies, no difficulties were experienced in transplanting single colonies to subsequent liver-veal agar plates. On transplanting a regular round colony there was obtained without difficulty, with some strains at least, a pure culture of the round colony form, identical with those obtained directly from the original non-sugar-fermenting culture.

Some strains, however, were extremely difficult to purify. Every time, even though a well isolated regular colony was taken, intermediates and even threadlike colonies appeared again after replating. Sometimes six successive transplants on liver-veal agar plates were necessary to eliminate both the intermediate and the threadlike forms.

The same procedure was necessary when attempts were made to obtain a pure culture of the threadlike colonies. No difficulty was experienced with some strains; by taking one of the most filamentous and well isolated colonies, a pure culture was obtained after one or two successive transplants. For some strains, however, not less than 12 successive transplants of single isolated colonies were necessary to eliminate the round and the intermediate colonies. This great number of transplants, necessary to obtain pure cultures of the regular as well as of the threadlike colonies is rather suspicious and indicates that we are not dealing with a simple contamination, which could have been removed easily with one or two transplants of single isolated colonies. It is highly improbable that every time, up to more than ten plates, a mixed colony is transferred. In this regard it has to be borne in mind that the modern technique, using McIntosh and Fildes anaerobic jars and a suitable culture medium (Difco-liver-veal agar) makes the work with the sporulating anaerobes scarcely more complicated than the work with most of the

aerobic bacteria. Practically without exception every plate contained many very well isolated colonies of 1 to 2 mm. in diameter.<sup>1</sup>

#### BIOCHEMICAL AND PATHOGENIC DIFFERENCES OF THE TWO TYPES

After obtaining pure culture plates of each strain of *C. histolyticum* from the regular as well as from the filamentous colony form, one colony from each plate was used for the inoculation of a fermentation medium, containing glucose, as well as for one without glucose. After two days incubation, the round colony form, from all of the 7 strains used, had not formed any acid or gas from the glucose, when compared with the blank without glucose. On the other hand all of the threadlike variants had formed considerable amounts of gas and acid, compared with the glucose-free blanks.

There is, thus, no doubt that the appearance of the fermenting ability after several transplants in tumor tissue suspension was due to the presence of organisms which gave rise to the threadlike colonies.

In this regard, attention may be called to the fact that Henry and also McIntosh and Fildes, who observed a sugar-fermenting ability of *C. histolyticum*, describe its colony form as delicate and flat, with crenated or irregular edges. Photographs given show that undoubtedly these investigators were dealing with colony forms intermediate between the perfectly round and the filamentous colony forms. When the sugar-fermenting ability of a number of such intermediate forms was tested during the present investigation, it was found that all were active, and that there was a certain correlation between the colony form and the amount of acid and gas produced from glucose. The more that

<sup>1</sup> After pouring the liver-veal agar plates, they were allowed to cool slowly. This prevented syneresis and usually the surface was dry after gelatination, so that the plate could be used immediately. Three one-inch-broad, parallel smears with a bent platinum needle were made over the surface, thus giving a continued dilution of deposited bacteria and leaving a space between the smears, which was of great value for detection of any contamination, as growth was allowed only on the surface touched with the needle. The dish was placed upside down in the jar with a piece of filter paper, wetted with three drops of glycerol in the cover; this gave the surface the right degree of moisture, but prevented creeping.



differentiation had proceeded towards the filamentous form, the more acid and gas were produced from glucose.

There is no doubt that the non-sugar-fermenting strains correspond closest to the original description of *C. histolyticum* given by Weinberg and Seguin. Since their identification is based mainly on their specific action on living muscle tissue, it was decided to test the pathogenicity of both the above types of colonies. On injecting 0.5 cc. of an 18-hour brain-medium culture intramuscularly into the shaved leg of a guinea pig, it was seen that the regular colony type caused the specific lysis of muscle tissue, as described by Weinberg and Seguin and Combiesco (1923) and others. The animals usually died within 48 hours after inoculation with any of the strains used. The filamentous colony form, on the other hand, was found to be relatively harmless. Sometimes a local oedema appeared after 24 hours without causing appreciable damage to the tissue, disappearing gradually afterwards. When injected, however, into necrotic tissue such as the inside of a large transplantable rat tumor, the whole tumor was usually liquefied within 48 hours with the production of a relatively large quantity of a hemorrhagic fluid with an offensive odor.

Another difference between the two types was the reaction in nutrient gelatin to which a strip of iron had been added. According to Spray (1936), *C. histolyticum* gives in this medium, 1 to 2 days after inoculation, a beautiful wine-red color, specific for this species, though the nature of the reaction is still unknown. In line with this, it was found that all tubes, inoculated with pure cultures of the regular colony form, gave this reaction, whereas those inoculated with the filamentous colonies did not. Intermediate forms gave intermediate reactions, more or less faint red, the color usually disappearing later. The color appeared only when growth occurred under semi-anaerobic conditions. By excluding all traces of oxygen, in an anaerobic jar, no red color appeared, even when, after growth had ceased, the tubes were placed in the air. Thus, it seems that traces of oxygen are necessary for the appearance of this reaction.

Finally, another difference between the biochemical properties of the regular and the filamentous colony types was found in

their conduct towards nutrient lead acetate agar. This medium was not blackened in 6 days by the regular colony type, whereas the filamentous form gave a smoky brown precipitate of lead sulfide in the same period of time.

#### IDENTITY OF THE FILAMENTOUS VARIANT WITH *C. SPOROGENES*

The foregoing facts undoubtedly indicate that the filamentous variants of *C. histolyticum* can be identified as *C. sporogenes*.

Inoculated in iron-milk, an inactive gaseous fermentation occurred, accompanied by a rapid digestion of the casein. Usually, in 48 hours, the medium was strongly blackened. Tyrosin was sometimes formed, though much less than that given by the original strains. Nutrient lead acetate medium was slightly blackened, but the color was not so dense as that usually given by *C. sporogenes*. Formation of indol was not observed during growth; however, a few strains (not all) gave, on reaction with a 5 per cent alcoholic vanillin solution and concentrated HCl, a positive "vanillin violet" test, a reaction also given by *C. sporogenes*. Gelatin was rapidly liquefied by the threadlike forms. Glucose and maltose were fermented, whereas lactose, sucrose and salicin were not. Threadlike colonies, similar to those obtained from the *C. histolyticum* strains, were also obtained from one authentic strain of *C. sporogenes* which was available. A still more convincing proof of the close relationship between the filamentous variants of *C. histolyticum* and *C. sporogenes* was the fairly good agglutination of these variants with serum of a rabbit which had been immunized against one strain of *C. sporogenes* which had been obtained from the American Type Culture Collection.<sup>2</sup>

#### METHODS FOR OBTAINING THE SUGAR-FERMENTING, SPOROGENES-LIKE VARIANTS OF *C. HISTOLYTICUM*

It must be borne in mind that *C. sporogenes* is probably the most common anaerobic contaminant of laboratory cultures, and

<sup>2</sup> More details about these agglutination reactions and other serological evidences of the close relationship of *C. histolyticum* and *C. sporogenes* will be given in a separate article by L. Smith to whom the author is much indebted for his interest in the problem.

great care must be taken that all possibility of contamination with this species be excluded. The gradual change, and the intermediate forms given are, however, sufficient proof that the filamentous colony form ultimately obtained is not due to contamination.

For the same reason, methods were sought which would give reproducible results with a minimum of manipulation. One of the methods devised is as follows:

A single isolated colony from a pure culture plate of *C. histolyticum* is inoculated into Difco-nutrient gelatin solution (pH = 7.3) to which a strip of stovepipe iron is added.<sup>\*</sup> Before inoculation, sterility is definitely established by incubation for at least 4 days at 37°C. in an anaerobic jar.

No seal is required, since the tubes must be incubated under semi-anaerobic conditions. Usually, within 16 hours after inoculation growth occurs, a wine-red color gradually developing which reaches its maximum intensity in 2 to 3 days.

When growth has been well established, and in less than 24 hours after inoculation, a transplant is made into a new medium regardless of whether color has appeared or not. This is repeated daily, thus enabling the bacteria to multiply for a considerable time under the most favorable conditions.

Gradually the growth becomes more abundant than in the initial tubes and the final wine-red color becomes paler. Ultimately the color fails to appear even after 3 days incubation, or else it appears on the first day, only to disappear on the following day.

When smears are made in this stage on liver-veal agar from tubes in the early stages of growth (at the time that a transplant to the new medium is made) colonies which are no longer perfectly round and which are sometimes more transparent than the others gradually appear (figs. 9-12). Furthermore, colonies with one or two thick, short shoots, also develop although, except on close examination under a dissection microscope, they do not appear markedly different from the others (figs. 2, 7 and 8).

<sup>\*</sup> More details as to the preparation of this medium may be found in the publication of Spray (1936) cited above.

These irregular outlines are the first signs of differentiation, and such colonies appear as soon as the red color no longer reaches its maximum intensity. Usually, when such a single separated irregular colony is replated on liver-veal agar, more than 90 per cent perfectly regular, round colonies appear. Of the remainder, nearly all appear like the original mother colony, while a few are more pronounced in their irregularity. By repeated selection and replating of one of the most irregular colonies on five or six successive plates, a pure culture of the threadlike variant is obtained.

Actually, the threadlike variants may be obtained more easily if the daily transplants in the iron-gelatin medium are continued beyond the appearance of the first signs of differentiation. Usually when the transplants in this medium are continued, the differentiation becomes more pronounced. Even though no red color is observed in a number of successive transplants there is always still a majority of the original regular colonies to be seen in the smear obtained from the final tube of such a series. In order to obtain a pure culture of the hairy colonies selective transplants on solid medium were always necessary. It was observed sometimes that the red color reappeared and the irregular colony forms disappeared again, when making subcultures every two or three days instead of within 24 hours.

When, to a pure culture of *C. histolyticum*, was added artificially an initial, very slight contamination of *C. sporogenes*, and a similar procedure was followed as with the pure culture, even the first transplants gave a great number of very hairy colonies and no intermediates by making smears on liver-veal agar. Moreover, the red color was still undiminished. This striking difference from a pure culture series makes it improbable that a contamination is responsible for the effect described.

The gelatin medium was by no means the only medium on which differentiation was obtained. This medium has the advantage, however, that the progress of the dissociation can be followed macroscopically. However, similar results may be obtained (usually even after a smaller number of transplants) by an analogous procedure in milk or in brain medium, and especially

in tumor-tissue suspension. It is probable that this differentiation could occur in any medium which is rich in suitable proteins. It is not even necessary to use a liquid medium. Continued transplants on liver-veal agar of single, separated colonies (if possible the most irregular ones) lead to the same result, though usually more transplants are required. All strains of *C. histolyticum* are much more resistant toward variation when the transplants are made on media containing only a restricted amount of peptones. The better the nutrient conditions, the more the variation proceeds in the direction of the sugar fermenting variants. The poorer the medium, the more the original properties of *C. histolyticum* are maintained.

Even on protein-rich media, such as liver-veal agar, it was observed that areas in the smear with a high population had less pronounced irregular colony shape, whereas those at the margins or decidedly isolated colonies, having the best nutrient conditions, were the most markedly irregular.

For the same reason it also seems advisable to use as a stock medium for *C. histolyticum* a 1 per cent peptone solution, solidified by the addition of 0.5 per cent agar, instead of the usual stock media, brain or Robertson medium.

The gradual change from the perfectly round and regular colony form of *C. histolyticum* to the hairy colonies, identical with those of *C. sporogenes*, is shown for one strain in figures 1 to 6.

This variation has been accomplished on liver-veal agar plates by replating each time the most irregular single, separated colony. In 9 transplants, the whole transformation was completed from the original *C. histolyticum* to a variant practically identical with *C. sporogenes*.

#### EXPERIMENTS TO OBTAIN THE ORIGINAL STRAIN FROM THE VARIANTS

Practically no difficulties in transforming variant forms into the round colony type were encountered when starting with an intermediate colony form, giving a faint though decided sugar fermentation. When a single colony from such a plate was replated on new solid media, there appeared usually, even on

the first transplant, perfectly regular colonies, besides many irregular ones. When one of these round colonies was used for a succeeding plate all intermediate forms gradually disappeared after several transplants, although sometimes 5 to 10 transplants of a single regular colony from successive agar plates were necessary. The farther variation had proceeded, the more difficult it was to obtain the original non-sugar-fermenting strain.

Consistent results were not obtained on liver-veal agar with the extremely filamentous colonies, although on several occasions a marked regression was observed after continued selection.

In general the best results were obtained by cultivation on poor media, as for example, 1 per cent or even less bacto-peptone agar, thinly poured. On this medium the colonies remained very small, but with the help of a dissection microscope, it was possible to transplant single separate colonies to new media of the same composition. Usually a considerable number of such plates was necessary to obtain regular round colonies.

It was observed that the sporogenes-like variants of *C. histolyticum* are like the original strain in that they are not strictly obligate anaerobes. On meat infusion agar, they form very minute colonies, scarcely visible, under aerobic conditions. It was possible to make several successive transplants.

#### DISCUSSION

Hitherto, we have always considered the filamentous sporogenes-like colony forms, obtained from pure cultures of *C. histolyticum*, as "variants" of the latter. It is questionable, however, whether it is not more reasonable to consider *C. histolyticum* as the variant, originating genetically from *C. sporogenes*. Indeed, variants are usually obtained as the result of a prolonged continuance of conditions *unfavorable* for normal growth (presence of phage, chemicals, antiserum, etc.) whereas growth under optimal conditions generally favors stabilization. In this respect it may be remembered that the better the nutrient conditions the more the *C. histolyticum* tends to give the sugar fermenting, filamentous sporogenes-like colonies. On the other hand, cultivation on poor media of the latter colony form tends to change

them back again to the original streptococcuslike colony form of *C. histolyticum*.

This does not mean of course, that every strain of *C. sporogenes* can be changed into a pathogenic, non-saccharolytic strain of *C. histolyticum*. However, on the other hand, it seems that although the conclusion here made is based on experiments with only seven different strains, every strain of *C. histolyticum* may be induced to undergo a change, which ultimately leads to a variant identical with *C. sporogenes*.

The different strains of *C. histolyticum* used undoubtedly showed different resistance, but using suitable methods, this resistance could be broken. It is practically broken as soon as irregular colonies appear on liver-veal agar plates, e.g., as represented in figures 7 to 12. Selection on this solid medium always leads gradually to the hairy colony form of *C. sporogenes*.

It is not intended to propose that the designation of the species *C. histolyticum* be changed to one such as *C. sporogenes*, var. *histolyticus*. Under usual conditions, the biochemical properties of *C. histolyticum* are markedly different from those of *C. sporogenes*, and, in addition, are stable enough to justify the collection of all nonsaccharolytic, pathogenic strains, with regular, streptococcuslike colonies, and giving a wine-red color in nutrient iron-gelatin, in the species *C. histolyticum*.

On the other hand, it must be borne in mind that, where variation can occur in morphology as well as in colony form or in biochemical behaviour, the three most important foundations on which classification of micro-organisms is based, the possibility is open that related species, which hitherto have been considered as distinctly different from each other, are nothing else than stabilized variants of the same species.

*Bacillus undulatus* and *Bacillus mycoides* (den Dooren de Jong, 1933) are one example, *C. histolyticum* and *C. sporogenes* are another and there are evidences that more will be found in the near future.

The author wishes to express his thanks to Dr. Ellice McDonald, Director, for his interest and support throughout this work.

## SUMMARY

1. *Clostridium histolyticum* on liver-veal agar grows in perfectly round, streptococcuslike colonies, is nonsaccharolytic, thoroughly pathogenic, and produces a wine-red color when inoculated into nutrient iron-gelatin.

2. Repeated early transplants on media rich in suitable proteins, allowing multiplication under the most favorable conditions for a considerable time, lead to the appearance of colonies with irregular shape.

3. Continued selection of the most irregular colonies on successive liver-veal agar plates, leads gradually to pure cultures of a filamentous colony form.

4. Gradual change in colony form corresponds with gradual change in biochemical properties: the appearance of sugar-fermenting ability and H<sub>2</sub>S formation, and the loss in pathogenicity and the ability to form the wine-red color in iron-nutrient gelatin.

5. The pure culture of these hairy variants is practically identical, biochemically, as well as in colony form, with *Clostridium sporogenes*.

6. To obtain the original strain of *Clostridium histolyticum* from the filamentous variants, continued selection on poor media was found to give the best results: this transformation could not always be obtained.

7. From the results of this study it would appear that there is a genetic relation between *Clostridium sporogenes* and *Clostridium histolyticum*.

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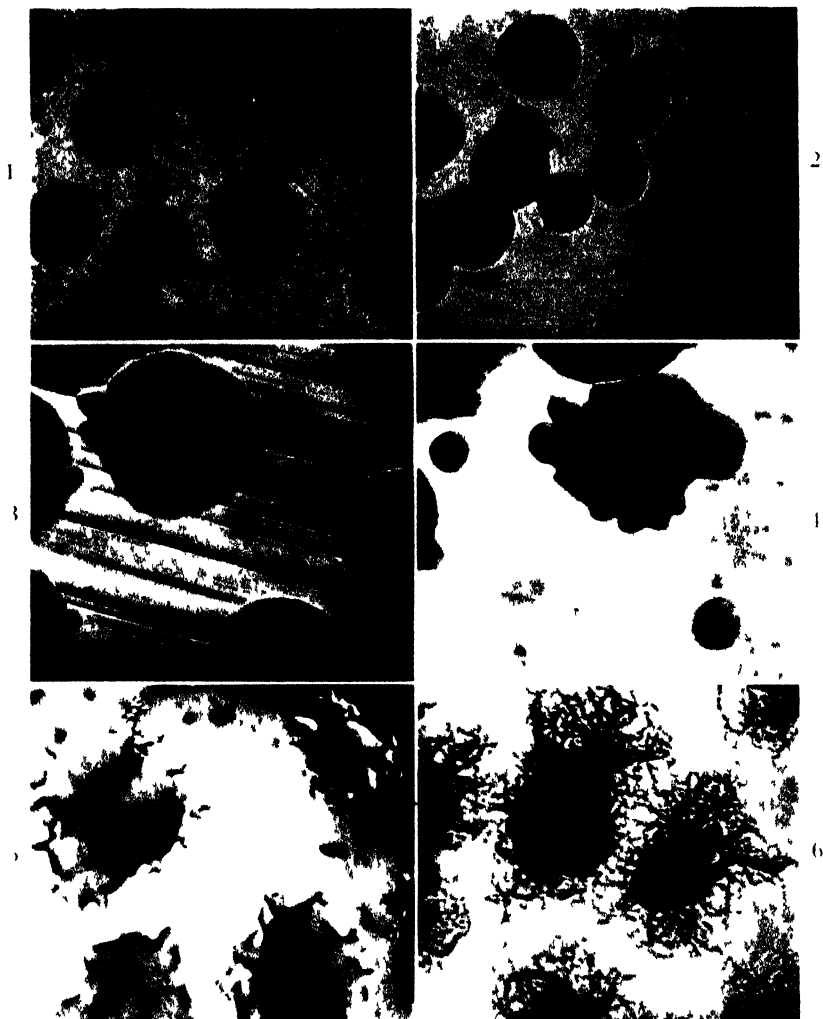


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## PLATES

## PLATE 1

Figures 1 to 6 show some phases of the gradual change from the round, streptococcus like colonies of *C. histolyticum* to the hairy ones of a variant, identical with *C. sporogenes*. A series of daily transplants of single, separated colonies was made on liver-veal agar plates. Figure 2 shows the appearance of the first signs of differentiation.



(1) (2) (3) (4) (5) (6) *Proteus mirabilis* flagella

## PLATE 2

Figures 7 to 12 give the results of smears on liver-veal-agar from tubes with brain medium, non-gelatin medium, milk and rat tumor suspension, media in which by daily transplants the first signs of differentiation occurred

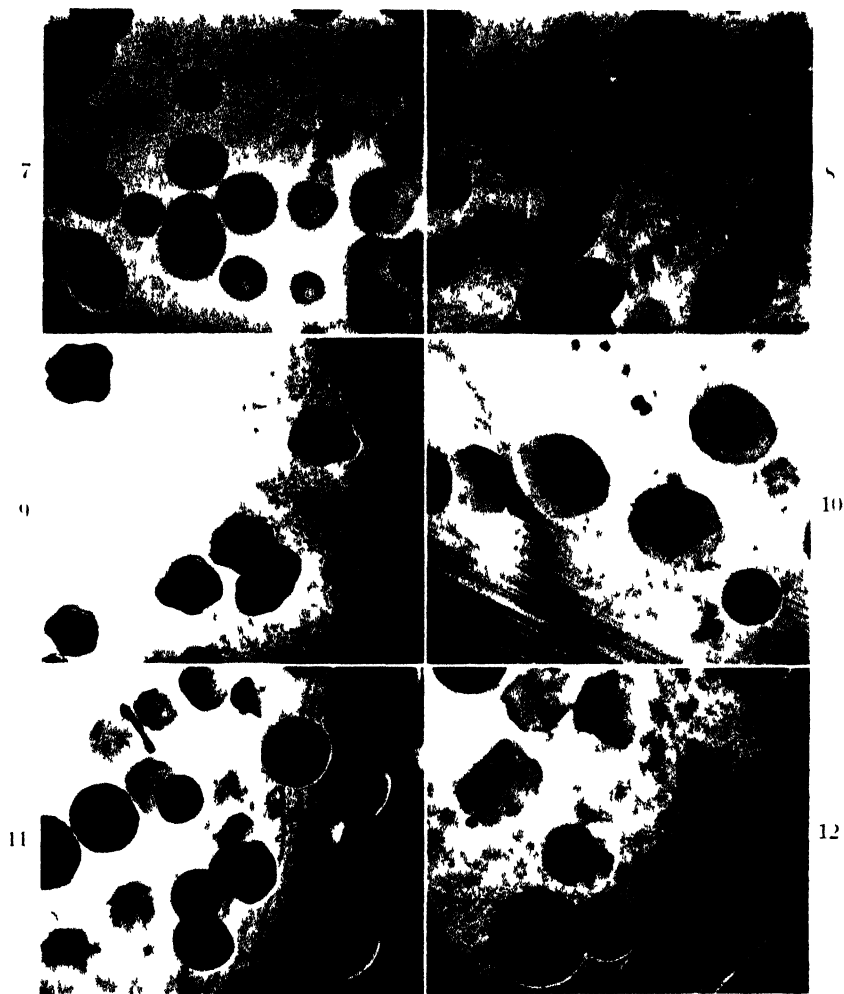


FIG. 2. Cellular structure of *B. subtilis* spores.



# SOME SEROLOGICAL ASPECTS OF THE S-R CHANGE IN CLOSTRIDIUM HISTOLYTICUM

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The variation in antigenic structure associated with the S-R change in aerobic pathogens seems to be fairly uniform. In general, there is a loss of the antigenic component characterizing the somatic surface of the bacteria in the smooth phase. This antigenic component is often, but not always, of the nature of a complex polysaccharide. The loss of the latter polysaccharide from the surface of the bacterial cells, whether present in the form of a capsule or not, renders the organism non-pathogenic and agglutinable not only by its own antiserum, but also by antiserum prepared against bacteria in the smooth phase, and sometimes by antisera prepared against rough forms of other species (Topley and Wilson, 1936).

The variation in the antigenic structure of the spore-bearing anaerobes has not been studied as fully, but it appears that the problem may be more complicated in this group. Orr, Josephson, Baker, and Reed (1933), working with *Clostridium welchii*, found that an antiserum prepared by the injection of bacilli in the smooth phase would not agglutinate the rough variant of the same culture. Colef (1936) isolated two non-toxic variants of *Clostridium oedematiens* which formed smooth colonies. Antisera prepared against these variants agglutinated the original culture to titer. Conversely, the antiserum prepared against the original culture agglutinated these variants. This cross-agglutination was probably not due to similar flagellar agglutinogens, for flagella could not be demonstrated on the variants, which were non-motile.



In the light of these facts, it was thought of interest to investigate the agglutination reactions and change of antigenic structure involved in the S-R change of *Clostridium histolyticum*.

#### METHODS

Of the strains of *C. histolyticum* used in this work, A and B were obtained from Dr. Weinberg of the Pasteur Institute, Paris; 4972 and 7263 were obtained from the American Type Culture Collection. Strain 4972 seemed to be primarily in the rough phase and the rough variants of the others were obtained by colony selection (Hoogerheide, 1937). All of the strains of *Clostridium sporogenes* were obtained from the American Type Culture Collection. The organisms were grown on liver-veal infusion agar (Spray's medium) in McIntosh-Fildes jars, at 37°C., for 24 hours.

Each plate was inoculated from a single, isolated colony. After incubation, the plates were examined under a dissection microscope and any intermediate colonies were removed. In the preparation of suspensions of the organisms in the smooth phase, which were to be used for immunization, two streaks were made with a platinum loop the full width of the petri dish and the bacteria were inoculated into semi-solid glucose agar containing brom-thymol-blue. These tubes of agar were incubated at 37°C. for 24 hours, at which time it was possible to ascertain that there was no acid formation, and hence, extremely slight, if any, contamination with rough variants. The bacteria on the plates, which in the meantime had been stored in the refrigerator, were suspended in 0.85 per cent NaCl and washed three times by centrifuging. The suspensions were filtered through cotton to remove clumps.

The antisera were prepared by the injection of healthy, male rabbits with a fresh suspension of the washed organisms containing 500 million bacteria per cc. This was administered intravenously in doses of 1, 2, and 3.5 cc., at 5-day intervals. Five days after the third injection, the rabbits were bled from the heart, the blood allowed to clot, and the serum was separated. One-hundredth of one per cent Merthiolate was added to the immune serum as a preservative.

Fresh suspensions for the agglutination tests were prepared daily. Agglutination was carried out at 56°C. for 2 hours. The tubes were allowed to stand overnight at 4° to 5°C. before the final readings were taken. In each set of agglutinations, a control tube, containing equal parts of the bacterial suspension and normal rabbit serum diluted 1:20, was incubated with the others as a control for any tendency toward spontaneous agglutination on the part of the organisms used.

In the course of the agglutination experiments, it was found of importance to use a young culture of the organisms in the smooth phase, since 48-hour cultures sometimes agglutinated slightly with antiserum prepared against organisms in the rough phase. The same phenomenon occurred if there was more than a trace of oxygen in the anaerobic jar, or if the suspensions of bacteria were allowed to stand at room temperature overnight.

#### EXPERIMENTAL

Antiserum prepared against the rough variant of strain 4972 was used to agglutinate smooth and rough variants of strains A, B, 4972, and 7263 (table 1). The absence of agglutination of the organisms in the smooth phase indicated that there had been at least two changes in the antigenic structure associated with the S-R variation: a loss of a somatic surface antigen, and a probable change in the antigenic structure of the flagella.

Because of the marked resemblance of the rough variants of *C. histolyticum* to *C. sporogenes*, various cultures of this latter organism were tested for agglutination against this antiserum. The titre to which these various strains agglutinated agreed with the results of Hall and Stark (1923) who found that strains 46, 94, and 115 agglutinated much better with serum prepared against heterologous strains of *C. sporogenes* than did strains 54, 118, and 133. The agglutination of strain 319 of *C. sporogenes* (1:640) was as high as that of two of the rough variants of *C. histolyticum*. It appears that there may be a number of common antigens in the various strains of these two species. Heating the suspensions of *C. sporogenes* to 100°C. for 1 hour to destroy the flagellar agglutinogens did not appreciably alter the titer to which they were agglutinated by this antiserum.

An antiserum prepared against *C. histolyticum*, strain A in the smooth phase, agglutinated to titer the other strains in this phase (table 2). The agglutination to high titer of the rough variant of the homologous strain and strain 4972, and the comparatively slight agglutination shown by *C. sporogenes* indicated that these rough variants are true variants, indeed, and not chance contaminants. Strain specificity seemed to be uncovered

TABLE 1

*Agglutination of C. sporogenes and of smooth and rough variants of C. histolyticum by antiserum to a rough variant of C. histolyticum*

STRAIN USED	DILUTION OF SERUM									CONTROL
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
<i>C. histolyticum</i> :										
A Smooth. . . . .	—	—	—	—	—	—	—	—	—	—
B Smooth. . . . .	—	—	—	—	—	—	—	—	—	—
4972 Smooth. . . .	—	—	—	—	—	—	—	—	—	—
A Rough. . . . .	++++	++++	+++	++++	++	+	—	—	—	—
B Rough. . . . .	++++	++++	+++	++++	+	—	—	—	—	—
4972 Rough. . . . .	++++	++++	++++	++++	++++	++	++	+	—	—
7263 Rough. . . . .	++++	++++	++++	++++	++	+	+	—	—	—
<i>C. sporogenes</i> :										
319. . . . .	++++	+++	+++	++	++	+	—	—	—	—
46. . . . .	++	++	+	+	—	—	—	—	—	—
54. . . . .	—	—	—	—	—	—	—	—	—	—
94. . . . .	++	+	—	—	—	—	—	—	—	—
115. . . . .	++	+	+	—	—	—	—	—	—	—
118. . . . .	++	+	—	—	—	—	—	—	—	—
133. . . . .	—	—	—	—	—	—	—	—	—	—

The figures in the table indicate the amount of agglutination in each tube; ++++ indicates complete agglutination; +++ almost complete; ++ partial; + slight.

by the S-R change in this organism, as shown by the difference in the titer to which the smooth and rough variants of strain B agglutinated with this antiserum. The complete agglutination exhibited by the organisms of this strain in the smooth phase, and the poor agglutination shown by those in the rough, can hardly be interpreted in any other way.

An antiserum was prepared against strain 319 of *C. sporogenes*.

TABLE 2  
*Agglutination of C. sporogenes and of smooth and rough variants of C. histolyticum by antiserum to a smooth strain of C. histolyticum*

STRAIN USED	DILUTION OF SERUM								CONTROL
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
<i>C. histolyticum</i> :									
A Smooth.....	+	+++	+++	+++	+++	+++	+++	+++	++
B Smooth.....	+++	+++	+++	+++	+++	+++	+++	+++	+
7263 Smooth.....	++	+++	+++	+++	+++	+++	+++	+++	+
A Rough.....	+++	+++	+++	+++	+++	+++	+++	+++	++
B Rough.....	++++	+++	+++	+++	-	-	-	-	-
4972 Rough.....	+++	+++	+++	+++	+++	+++	+++	+++	-
<i>C. sporogenes</i> :									
54.....	-	-	-	-	-	-	-	-	-
94.....	+++	++	+	-	-	-	-	-	-
133.....	-	-	-	-	-	-	-	-	-
115.....	-	-	-	-	-	-	-	-	-
118.....	-	-	-	-	-	-	-	-	-
319.....	+++	++	++	++	+	-	-	-	-

This was tested against suspensions of the variants of strains A and B (table 3). The non-agglutination of the organisms in the smooth phase, and the agglutination to low titer of those in the rough, were in accordance with the results reported above.

Since the results of these agglutination tests pointed toward the existence of an antigen on the somatic surface of the smooth bacilli which was not to be found on the rough variants, an attempt was made to isolate it. Extractions of thrice-washed bacteria were made with N/20 NaOH and N/20 HCl. The acid extraction gave superior results. Preliminary precipitation experiments indicated that this acid extract contained a substance which reacted with antisera prepared against organisms in the

TABLE 3

*Agglutination of smooth and rough variants of C. histolyticum by antiserum to C. sporogenes, 319*

STRAIN USED	DILUTION OF SERUM									CON- TROL
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
<i>C. sporogenes</i> 319.	-	++	++	+++	++++	++++	++++	-	-	-
<i>C. histolyticum</i> :										
A Rough.....	++++	++++	+++	++	++	+	-	-	-	-
B Rough.....	+	+	++	++	+	-	-	-	-	-
A Smooth.....	-	-	-	-	-	-	-	-	-	-
B Smooth.....	-	-	-	-	-	-	-	-	-	-

smooth phase, and that this substance was precipitated by 75 per cent alcohol, but not by trichloroacetic acid or full saturation with ammonium sulfate. Since this is what would be expected of a complex polysaccharide, an attempt was made to prepare it by methods used in the isolation of polysaccharides.

Four liters of 3 per cent Neopeptone, adjusted to pH 7.2, were inoculated with *C. histolyticum* strain A, in the smooth phase, and allowed to incubate for 36 hours. The bacteria were centrifuged, washed three times with 0.85 per cent NaCl, and heated at 80°C. for 10 minutes with ten times their volume of N/20 HCl. The mixture was centrifuged, the supernatant poured off and saved, and the extraction repeated. The supernatant was added to that from the first extraction, the mixture of the two brought

to pH 7.0 with 1 N NaOH, centrifuged, and the precipitate discarded. To the supernatant were added four volumes of absolute alcohol, and this mixture kept overnight in the refrigerator. The precipitate, obtained by centrifuging, was added to 20 cc. of 30 per cent KOH and heated at 100°C. for two hours. It was then neutralized with a saturated solution of trichloroacetic acid, centrifuged, and the precipitate discarded. The supernatant fluid, after being dialyzed against running tap water for 48 hours, was added to four volumes of absolute alcohol and allowed to stand in the refrigerator. It was then centrifuged, the supernatant discarded, and the precipitate, a fine white powder, was dried. Some of it was dissolved in saline to give a concentration of 1:1500 and portions of this solution, undiluted and diluted 1:10, 1:100 and 1:1000, were set up in precipitin tests against

TABLE 4

*Precipitin tests of polysaccharide with rough and smooth antiserum*

	DILUTION							
	—		1:10		1:100		1:1000	
	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth
Antiserum.....								
Precipitation.....	±	+++	—	+++	—	++	—	++

antisera to rough and smooth *C. histolyticum*. These were incubated at 37°C. for two hours, and were stored overnight at 4° to 5°C. before the final reading was made (table 4).

Although this white powder gave a slightly positive Biuret test, it was assumed that it was primarily carbohydrate in nature. The Molisch test was positive, the xanthoproteic test negative. The slightly positive Biuret test was no doubt due to protein breakdown products resulting from the hydrolysis with KOH, although most of these substances should have been removed by the trichloroacetic acid precipitation and the dialysis. It gave a precipitate with a 0.5 per cent solution of the reduced base of crystal violet (4, 4', 4'' hexamethyltriaminotriphenyl methane) and the reduced base of malachite green (4, 4'' tetramethyldiaminotriphenyl methane). These two reagents were reported by Chapman and Lieb (1937) to give precipitates with a polysac-

charide isolated from staphylococci, but not with polysaccharides non-bacterial in nature.

#### DISCUSSION

This study yielded evidence that the rough variants of *Clostridium histolyticum* were true variants, and not chance contaminants of *Clostridium sporogenes*. It appeared, however, that there are a number of antigens common to both *Clostridium sporogenes* and *Clostridium histolyticum*. These antigens did not enter into agglutination reactions of the normal smooth *Clostridium histolyticum*, for they apparently were masked by the somatic surface antigen. When the smooth strains underwent a S-R change, the surface antigen was lost and the underlying, less specific, antigens then formed the somatic surface and came to play an important part in agglutination reactions.

Hall and Stark (1923) and Starin and Dack (1923) found that *Clostridium sporogenes* would not agglutinate with antisera to a number of other spore-bearing anaerobes, nor would these organisms agglutinate with anti-*Clostridium sporogenes* serum. Consequently, *Clostridium sporogenes* must be more closely related to *Clostridium histolyticum* than to *Clostridium welchii*, *Clostridium botulinum*, *Clostridium oedematis-maligni*, *Clostridium bifermentans*, *Clostridium centrosporogenes*, *Clostridium butyricum*, *Clostridium tetani*, or *Clostridium tyrosinogenes*, which were the organisms used by the former workers. It appeared, from the agglutination reactions of *Clostridium sporogenes* 319 and the agglutination reactions of the rough variant of *Clostridium histolyticum* strain B, that it may be impossible to differentiate by simple agglutination between a typical strain of *Clostridium sporogenes* and certain stable rough variants of *Clostridium histolyticum*.

#### SUMMARY

1. Normal smooth strains of *Clostridium histolyticum* did not agglutinate with an antiserum prepared against a rough variant.
2. Rough variants, and certain strains of *Clostridium spor-*

*ogenes*, agglutinated with antisera to the normal smooth *Clostridium histolyticum* or the rough variants.

3. Rough variants agglutinated, although not to full titer, with antiserum to *Clostridium sporogenes* 319.

4. The smooth strains of *Clostridium histolyticum* possessed a complex polysaccharide as a somatic surface antigen.

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## STUDIES ON ANAEROBIC BACTERIA

### XII. THE FERMENTATION PRODUCTS OF *CLOSTRIDIUM THERMOSACCHAROLYTICUM*<sup>1</sup>

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*Clostridium thermosaccharolyticum*, produces acid and gas from a variety of carbohydrates and causes a "hard swell" type of spoilage in canned foods. The literature was reviewed and the organism described by McClung (1935 a and b). The lack of definite information on the fermentation of carbohydrates by pure cultures of thermophilic bacteria in general and the importance of this organism in the spoilage of canned food warranted a study of its fermentation products.

#### EXPERIMENTAL

Two pure strains of *C. thermosaccharolyticum*, no. 5 and no. 20, were used. Culture 20 was, moreover, obtained by single-cell isolation. Since both cultures gave very similar results, they will not be differentiated in this report. The stock cultures were kept in corn-liver medium and transfers were made to liver-infusion broth to obtain active cultures for inocula. Unless otherwise stated, all cultures were incubated at 55° to 60°C.

Favorable conditions for sugar utilization were first determined. In a 1-per-cent tryptone medium less than 10 per cent of the sugar was fermented. When calcium carbonate and reduced iron were added, sugar utilization increased considerably but was complete only when the original concentration of glucose was 1 per cent. In an attempt to ferment completely higher concentra-

<sup>1</sup> This work has been aided by a grant from the Wisconsin Alumni Research Foundation.

tions of glucose, liver extract, yeast extract, asparagine, dibasic ammonium phosphate and an inorganic salt mixture were used as supplements to the tryptone. Fermentation was not appreciably increased in any of these media. Two-per-cent malt sprouts, however, was found to be a good medium when an excess of calcium carbonate was present. Since other additional nitrogen sources had little effect upon the utilization of the sugar, it appeared that the influence of malt sprouts might be a "surface effect." This possibility was suggested by an experiment in which a water extract of malt sprouts and the same plus asbestos were compared. The extract alone was ineffective, but a combination of the two was as good as malt sprouts. Tryptone with asbestos also served as an adequate medium, but fermentation was not always so consistent as in the malt sprouts. The favorable influence of particulate matter on the growth of bacteria in liquid media has been suggested as being due to: lowering the oxidation-reduction potential of the medium, supplying surface for the organisms, and adsorption of nutrients. In addition, the malt sprouts or the asbestos also prevents caking of the carbonate in the bottom of the flask and thus aids in the neutralization of acids.

In all of the following fermentations approximately 5 cc. of an actively growing culture were inoculated into 300 cc. of 2-per-cent malt sprouts medium containing 3.5 grams of calcium carbonate and approximately 6 grams of glucose (sterilized separately).

#### *Identification of the products of the fermentation*

The gas evolved was bubbled through potassium hydroxide and the remainder was collected over water. The presence of carbon dioxide was verified by the partial neutralization of the potassium hydroxide solution by the gas and the precipitation of barium carbonate when barium chloride solution was added. The remaining gas was shown to be hydrogen by the combustion test.

No acetylmethylcarbinol or 2,3-butylene glycol was found. Neutral volatile products were determined on a 50 cc. distillate from 200 cc. of fermentation liquor made alkaline to phenol-

phthalein. This distillate contained no significant amount of butyl alcohol, ethyl alcohol, acetone or isopropyl alcohol.

Volatile acids were steam-distilled from an acid solution and identified by the Duclaux method as modified by Virtanen and Pulki (1928). By two methods of calculation, good agreement was obtained for acetic and butyric acids. Formic acid was absent according to the method of the Association of Official Agricultural Chemists (1930).

The non-volatile acid fraction remaining from the steam distillation was extracted for 48 hours with ethyl ether. The extract was taken up in water and an aliquot, titrated with barium hydroxide, showed a large amount of non-volatile acid. Succinic acid was absent as shown by the failure to obtain silver succinate on addition of silver nitrate to the neutral solution. Since the solution gave a positive thiophene test for lactic acid, the zinc salt of the acid was prepared and analyzed. The water of crystallization of the salt was found to be 18.23 per cent, corresponding to the calculated value of 18.17 per cent for inactive zinc lactate. Polariscopes readings also showed that the salt was inactive. The zinc oxide content of the anhydrous salt was 33.46 per cent compared with the theoretical value of 33.42 per cent. Ninety-six per cent of the theoretical quantity of lactic acid in the zinc lactate was obtained by the method of Friedemann and Graesser (1933). The identity of the lactic acid was confirmed by preparation of the para-phenyl-phenacyl ester which melted at 141°C. compared with that of known lactic acid which melted at 141.5°C. The mixed melting point was 141°C.

#### *Methods of quantitative analysis*

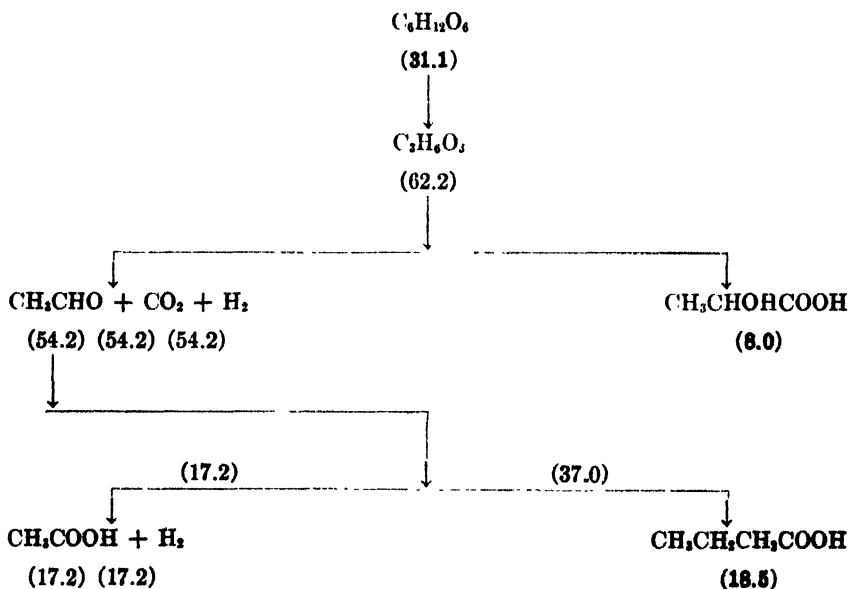
When gases were to be determined, the carbon dioxide was absorbed in alkali and the remaining gas was collected over water and calculated as hydrogen. The gas dissolved in the fermentation liquor was removed by aspiration with carbon-dioxide-free air. The carbon dioxide produced was calculated from the difference between that of the fermented culture and an uninoculated control.

Sugar was determined by the method of Stiles, Peterson and

Fred (1926). The Virtanen and Pulki (1928) distillation method was used for the determination of volatile acids. Lactic acid was extracted with ether and determined by the method of Friedemann and Graeser (1933).

### *Fermentation of glucose*

The dissimilation of glucose by *C. thermosaccharolyticum* was investigated by analyses of fermentations after incubation periods of 5 days at 58°C. Table 1 shows the results of a typical fermentation of glucose in which all products were determined. The weight, carbon and oxidation-reduction balances in each case approach the theoretical values, indicating that all major products have been found and that the determinations were made with relative accuracy. On the basis of these data, the breakdown of glucose by *C. thermosaccharolyticum* might be represented as follows:



The figures in bold-face are millimols found; the others were calculated. A comparison of the calculated values with those found is as follows:

	Calculated mM	Found mM
Carbon dioxide . . . . .	54.2	54.7
Hydrogen . . . . .	71.4	71.4
Acetic acid . . . . .	17.2	15.1

The agreement is satisfactory and apparently substantiates the above scheme of dissimilation.

TABLE 1

*Products of glucose fermentation by C. thermosaccharolyticum*

	WEIGHT		CARBON	OXIDIZED	REDUCED
	mgm.	mM	mM	mM	mM
Glucose used . . . . .	5600	31.1	186.7		
Products:					
Carbon dioxide . . . . .	2405	54.7	54.7	109.3	
Hydrogen . . . . .	144	71.4			71.4
Acetic acid . . . . .	908	15.1	30.2		
Butyric acid . . . . .	1625	18.5	74.0		37.0
Lactic acid . . . . .	720	8.0	24.0		
Total . . . . .	5802		182.9	109.3	108.4
Per cent recovery . . . . .	103.4		98.0		
O/R . . . . .					1.008

### *The effect of temperature upon the fermentation*

Since *C. thermosaccharolyticum* grows over a rather wide range of temperature, a series of cultures in glucose malt-sprouts medium were incubated at 37°, 45°, 55° and 65°C. The results of the analyses are reported in table 2. Practically all of the glucose was fermented at 45° and 55°C., lesser amounts at 37° and 65°C. Upon expression of the products in per cent of the glucose fermented, an interesting relation is observed. The volatile acids increased with increase in temperature. A higher percentage of the total appeared as acetic at the higher temperatures as shown by the increase in the ratio of acetic to butyric. The per cent of the glucose appearing as lactic acid and the total acidity decreased with increase in temperature. There was evidently a corresponding increase in gas formation.

Since fermentation was much more active at the higher temperatures, it was postulated that perhaps the rapid evolution of

gas would remove oxygen from the flask and in that way affect the final products. Cultures were, therefore, set up with mercury

TABLE 2

*The effect of temperature on products of glucose fermentation*

INCUBATION		GLUCOSE USED		PER CENT OF THE GLUCOSE FERMENTED				RATIO Ac/Bu
Temperature	Time	Milli-grams	Per cent	Acetic	Butyric	Lactic	Total	
With cotton plug								
°C.	days							
37	15	4020	65.1	3.1	10.6	71.2	84.9	0.288
45	15	6080	98.4	5.9	11.0	66.5	83.4	0.539
55	15	6090	98.5	6.5	13.8	56.9	77.2	0.476
65	15	4460	70.6	13.3	17.4	35.2	65.9	0.768
55	5	6025	90.2	4.8	11.8	56.1	72.7	0.408
With carbon dioxide and mercury seal								
37	18	5975	97.2	11.2	29.2	25.2	65.6	0.384
45	18	6065	98.6	18.1	35.9	8.1	62.1	0.505
55	20	6165	97.4	16.5	34.8	3.7	55.0	0.473
55	5	6385	95.5	15.2	27.4	10.8	53.4	0.555

TABLE 3

*Products of xylose fermentation by C. thermosaccharolyticum*

	WEIGHT		CARBON	OXIDIZED	REDUCED
	mgm.	mM	mM	mM	mM
Xylose used . . . . .	5845	38.8	193.8		
Products:					
Carbon dioxide . . . . .	2497	56.7	56.7	113.4	
Hydrogen . . . . .	117	58.2			58.2
Acetic acid . . . . .	800	13.2	26.7		
Butyric acid . . . . .	1688	19.2	76.7		38.4
Lactic acid . . . . .	1117	12.4	37.2		
Total . . . . .	6219		197.3	113.4	96.6
Per cent recovery . . . . .	106.3		101.9		
O/R . . . . .					1.175

seals and carbon dioxide was bubbled through to remove the oxygen at the start. In these fermentations less lactic acid and

more volatile acids were formed at each temperature than in duplicate fermentations exposed to the air.

Because of slow fermentation at the lower temperatures, it was necessary to incubate the above cultures for a relatively long time for more accurate comparison of results. In order to determine the influence of a short incubation period on the products formed, two fermentations in this series were analyzed after 5 days at 55°C. In the flasks with cotton plugs, there was little difference in the two series. In the flasks with carbon dioxide and mercury seals, there was slightly more butyric acid and less lactic acid after 20 days than after 5 days. This small difference may, however, be due only to variation between individual fermentations.

### *Fermentation of xylose*

In order to compare the dissimilation of a pentose with that of the hexose reported above, duplicate fermentations of xylose were analyzed as previously described. The results of one of these are summarized in table 3. The same products appeared in about the same amounts as from glucose. Calculation of fermentation balances shows that the weight and carbon balances are good, but the oxidation-reduction balance is obviously too high. Similar results were obtained when the experiment was repeated. There is no evidence to indicate the formation of another product. Therefore, we can now offer no completely satisfactory explanation for this slight discrepancy. Some error may have resulted from impurities in the xylose which was prepared from corn-cob hydrolyzate. Optical rotation of the equilibrium mixture gave a value of  $[\alpha]_D^{23^\circ\text{C}} = +20.2$  compared with  $[\alpha]_D^{20^\circ\text{C}} = +19.0$  reported by Hudson and Yanovsky (1917).

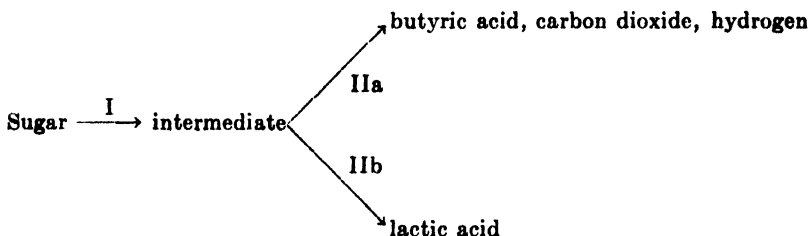
### DISCUSSION

In addition to the fact that its optimum temperature for growth is so high, *C. thermosaccharolyticum* is remarkable for its ability to produce both lactic and butyric acids from glucose. Although it is generally agreed that lactic-acid bacteria do not produce butyric acid, there have been reports of lactic acid production by



some of the so-called mesophilic butyric-acid bacteria (Hussong and Hammer, 1930-31; Kubowitz, 1934; Ris, 1936). *C. thermosaccharolyticum* differs, however, from a typical "butyric" in the formation of large proportions of lactic acid and no neutral volatile products.

The close relationship between the production of butyric and acetic acid by bacteria has also been demonstrated by Kubowitz (1934). In the presence of carbon monoxide the butyric-acid fermentation of *Clostridium butyricum* was inhibited and lactic acid became the principal product. Kubowitz suggested that, if the dissimilation of sugar be represented by:



the relative amounts of end products would depend upon the velocity constants of reactions IIa and IIb. In a normal butyric fermentation the velocity constant of reaction IIa is large, and little or no lactic acid is formed. If, however, reaction IIa is inhibited by carbon monoxide, the concentration of the intermediate increases to such a point that reaction IIb can proceed, and lactic acid is formed. That some such hypothesis might be applicable in the *C. thermosaccharolyticum* fermentation is indicated in the wide fluctuation in amounts of the various products formed under different conditions. In this case, reaction IIa would result in the formation of acetic acid in addition to butyric acid, carbon dioxide and hydrogen. We might then postulate that this reaction is inhibited by atmospheric oxygen with the result that lactic acid becomes the principal product of the fermentation.

Another possible explanation might be that the formed lactic acid is further utilized by the organism, especially under conditions of rapid growth at elevated temperatures or in the strict

absence of atmospheric oxygen. At any rate, the wide variation in the ratio of the acids formed, as shown in table 2, indicates that temperature, atmospheric oxygen and perhaps other factors, have a pronounced effect upon the manner of dissimilation.

#### SUMMARY

A malt-sprouts base medium with calcium carbonate appeared to be the most satisfactory of the several media tried for complete sugar utilization by *Clostridium thermosaccharolyticum*. The products of glucose fermentation were carbon dioxide, hydrogen, acetic acid, butyric acid and lactic acid. The ratio of products varied with change in temperature and appeared to be influenced by access of atmospheric oxygen to the fermentation. Neutral volatile products were either absent or present in very small amounts. Tests for formic acid, succinic acid, acetylmethylcarbinol and 2,3-butylene glycol were negative. The products formed from xylose were the same as those from glucose.

The author wishes to express his appreciation to Dr. Elizabeth McCoy, Dr. L. S. McClung and Dr. W. H. Peterson for helpful advice and criticism during the progress of this work and in the preparation of this manuscript.

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# NICOTINIC ACID AS A GROWTH ACCESSORY SUBSTANCE FOR THE DIPHTHERIA BACILLUS

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The last papers of this series, dealing with studies of the tissue extract requirements of the diphtheria bacillus for growth, described the separation of the essential substances contained in liver extract into an ether-soluble and ether-insoluble fraction (Mueller and Subbarow, 1937), and the identification of the former as pimelic acid (Mueller, 1937a). It is now possible to throw further light on the nature of the substances not extracted from acid aqueous solution by ether.

Because of the advantages of fractional distillation over methods of precipitation or adsorption, in dealing with the complex mixture which confronts one in a tissue extract, a means was sought of converting as much as possible of the extractive material into a form of sufficient stability to withstand vacuum distillation. Recalling the method suggested some years ago by Cherbuliez, Plattner and Ariel (1929; 1930) for distillation of amino acids after combined esterification and acetylation, it appeared possible that the same procedure might succeed with a somewhat different type of mixture. One would expect to obtain a very complex type of distillate, containing at least (1) simple esters of acids having no group subject to acetylation, (2) acetyl derivatives of amino or hydroxyl compounds, etc., but possessing no carboxyl group, and (3) combinations of the two. In any case, should one or more of the growth-promoting substances appear in such a distillate, careful fractionation might well permit a considerable degree of purification.

The writer was able, in 1932, to confirm the statements of Cherbuliez, Plattner and Ariel in regard to the yield and stability of distillates obtained by their method from protein hydrolysates; he prepared by partial hydrolysis with alkali, several acetylated amino acids in analytically pure form using redistilled fractions of such distillates. Some of these derivatives crystallize in a pure form much more readily than the amino acids themselves, into which they can easily be converted by acid hydrolysis. Since the work was carried out in connection with studies on the amino acids required for bacterial growth, it was not done in such a way as to be particularly suitable for publication, and has never been reported. Although, as far as can be learned, the method has been developed no further, either by its authors or by other workers, it appeared that it had many possibilities in connection with studies of amino acids, and that it might yield equally good results with many extractive materials. The greatest shortcoming of the method as used five years ago was the failure to obtain anything approaching pure compounds by repeated fractional vacuum distillation. Experience gained in carrying out the isolation of pimelic acid indicated the likelihood of much greater success in that part of the method by using a fractionating column such as that of Rittenberg.<sup>1</sup>

A preliminary experiment was carried out using 10 cc. of the alcoholic solution (4b) of the ether insoluble active fraction used in the control solution for the pimelic acid work. This contained about 0.4 gram solid material. The procedure followed was identical with that of Cherbuliez and his co-workers, except that the material was not refluxed either following saturation with HCl or after the addition of acetic anhydride and sodium acetate. The more strictly chemical details of this work will appear elsewhere (Mueller, 1937b).

Distillation of the resulting "acetylated esters," after removal of solvents and excess reagents, was carried out from a very small bulb with a slightly U-shaped side arm as receiver. An oil pump vacuum of about 0.04 mm. Hg was employed, and the temperature

<sup>1</sup> Personal communication.

of the bath carried to 200° before decomposition of the residue began to occur. The distillate weighed 0.233 gram, a very satisfactory proportion of the total material taken. It was a light yellow oil containing some solid indefinitely crystalline material.

The oil was drained from the solid as well as possible, and each was hydrolyzed by autoclaving with N/1 HCl at 10 pounds for 10 minutes. Tests were carried out by adding the material, as indicated, to the following control solution:

Casein hydrolysate (20 per cent)	0.5 cc.
Cystine	1.0 mgm.
Glutamic acid hydrochloride	50.0 mgm.
Lactic acid (as Na salt)	0.1 cc.
Pimelic acid	0.001 mgm.
Salt mixture	
Phenol red	

Quantities given are for 10 cc. medium.

Table 1 shows the effect of the addition of the hydrolyzed distillates to the control solution.

Since the addition of 0.01 cc. of the alcoholic 4b solution to 10 cc. of control medium permits the formation of 3.2 to 3.5 mgm. bacterial N, it is clear that the distillate carries a considerable proportion of the activity. A larger scale preparation was therefore undertaken.

Through the courtesy of Dr. Y. Subbarow of the Department of Biological Chemistry, Harvard University Medical School, and of the Lederle Company, Inc., Pearl River, New York, a concentrate of 300 kilograms of liver was made available. Briefly, this consisted in the 96 per cent alcohol soluble fraction of an aqueous extract, concentrated in vacuo until 1.0 cc. was equivalent to 100 grams liver tissue. Solid material, separating on long chilling, was removed by filtration, and the syrupy filtrate, after acidification, was extracted with isoamyl alcohol. Vacuum distillation of the extract with the occasional addition of water, removed most of the isoamyl alcohol, leaving about 1 liter of an aqueous solution. The extract was supplied in this form by the Lederle Company, to whom the writer wishes to express his appreciation and thanks.

The procedure followed in converting this material into its

acetylated esters is fully described elsewhere (Mueller, 1937b), and need not be repeated. It differs in no material respect from that recommended by Cherbuliez, Plattner and Ariel.

The first distillation was carried out rapidly from a Claissen flask, and the distillate was divided roughly into (a) low, (b) medium and (c) high boiling fractions by means of a Fischer triangle. The quantities obtained were respectively 14.8, 62.0 and 17.1 grams. Distillation was continued until the bath temperature reached 220°, at which point some decomposition of the residue began to take place.

TABLE 1\*  
*Effect of distillates of acetylated esters of "4b"*

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control + hydrolysate of solid portion, 0.02 mgm.	1.16
2	Control + hydrolysate of solid portion, 0.05 mgm.	1.74
3	Control + hydrolysate of solid portion, 0.1 mgm.	2.20
4	Control + hydrolysate of solid portion, 0.2 mgm.	2.88
5	Control + hydrolysate of liquid portion, 0.03 mgm.	2.66
6	Control + hydrolysate of liquid portion, 0.075 mgm.	3.02
7	Control + hydrolysate of liquid portion, 0.15 mgm.	3.30
8	Control + hydrolysate of liquid portion, 0.3 mgm.	3.54
9	Control	0.58

\* In the case of this particular experiment, the National Institute of Health strain of the Park 8 diphtheria bacillus was used instead of the Allen strain. The control was therefor enriched by 1.0 mgm. tryptophane and 0.05 cc. ethyl alcohol.

These fractions, after hydrolysis of small portions with N/1 HCl, when added to control media gave the results indicated in table 2.

Evidently the low boiling fraction alone is either without growth-promoting power, or contains an inhibitory substance. When mixed with the other fractions, the latter effect is indicated.

These distillates were now slowly re-distilled from the Rittenberg apparatus, modified by sealing on a glass bulb to permit dealing with large quantities. In this distillation, the original three fractions were expanded to thirteen. All of these were oils ex-

cept the second, which consisted of 3 to 4 grams of coarse white crystals, which were later identified as acetamide, resulting from the presence of ammonium salts in the extract. This fraction, very troublesome because of the obstruction of the side arm, which necessitated the use of a direct flame to keep the passage clear during distillation, could have been avoided by short alkaline vacuum distillation of the original extract to remove the  $\text{NH}_3$ .

Hydrolysis of a few milligrams of each of these with HCl permitted the tests of table 3 to be carried out.

In order to get additional information, each fraction was next tested over a wider quantitative range; then combinations of various fractions were tested in twos, attempting so to choose

TABLE 2  
*Effect of fractions of first distillate (Allen strain)*

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control + fraction (a), 1.0 mgm.	0 21
2	Control + fraction (b), 1.0 mgm.	2 22
3	Control + fraction (c), 1.0 mgm.	2 00
4	Control + fractions (a) + (b), 1.0 mgm. each	1 54
5	Control + fractions (a) + (c), 1.0 mgm. each	1 66
6	Control + fractions (b) + (c), 1.0 mgm. each	3 08
7	Control + fractions (a) + (b) + (c), 1.0 mgm. each	2 69

quantities of each that a mutual or simply an additive effect could be brought out. Then more than two fractions were combined in the same way. It would be quite useless to present these experiments in detail. Of course, no one of these fractions represents a pure compound. It is surprising how far below its boiling point a substance will begin to appear in a distillate, and how far above it, traces will still persist. Had there been any means of knowing the number of compounds in these distillates which either accelerate or inhibit the growth of our organism, and which ones would show an effect in the absence of the others, the analysis of these results would have been simplified. Without such knowledge, it was merely possible to conclude that the lowest and the highest fractions probably contained substances



inhibitory in concentrations of 1.0 mgm. or somewhat less, and that more than one, and possibly several accelerating substances were present.

Gradually it became evident that the most striking mutual effect was shown by combining suitable quantities of the low-

TABLE 3  
*Effects of fractions of second distillate*

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control + fraction 1, 1.0 mgm.	0.16
2	Control + fraction 1, 0.1 mgm.	0.14
3	Control + fraction 2, 1.0 mgm.	0.18
4	Control + fraction 2, 0.1 mgm.	1.26
5	Control + fraction 3, 1.0 mgm.	1.38
6	Control + fraction 3, 0.1 mgm.	0.42
7	Control + fraction 4, 1.0 mgm.	2.08
8	Control + fraction 4, 0.1 mgm.	0.52
9	Control + fraction 5, 1.0 mgm.	0.60
10	Control + fraction 5, 0.1 mgm.	0.10
11	Control + fraction 6, 1.0 mgm.	1.42
12	Control + fraction 6, 0.1 mgm.	0.32
13	Control + fraction 7, 1.0 mgm.	0.72
14	Control + fraction 7, 0.1 mgm.	0.28
15	Control + fraction 8, 1.0 mgm.	2.04
16	Control + fraction 8, 0.1 mgm.	0.68
17	Control + fraction 9, 1.0 mgm.	1.38
18	Control + fraction 9, 0.1 mgm.	0.40
19	Control + fraction 10, 1.0 mgm.	0.88
20	Control + fraction 10, 0.1 mgm.	0.24
21	Control + fraction 11, 1.0 mgm.	1.16
22	Control + fraction 11, 0.1 mgm.	0.28
23	Control + fraction 12, 1.0 mgm.	1.50
24	Control + fraction 12, 0.1 mgm.	0.20
25	Control + fraction 13, 1.0 mgm.	0.12
26	Control + fraction 13, 0.1 mgm.	0.32

boiling and of the high-boiling fractions. At this stage, too, the crystals making up the greater part of the second fraction were identified as acetamide, representing simply an inert impurity. The substance was present in solution also in fractions 1, 3 and 4. These fractions were consequently re-distilled using a special

receiver which enabled the side arm of the distilling column more readily to be kept free from solid material. After concentrating the acetamide as completely as possible into a single fraction, it was recrystallized from chloroform, the mother liquors containing the active substance added to the fluid fractions, and again fractionally distilled, this time very slowly in an attempt to obtain the active material in as pure a state as possible.

Acetamide was still present, however, in sufficient quantity to separate on standing in the ice box from the two lowest boiling fractions. These likewise contained the highest concentration of active material.

Meanwhile, all the other middle and high-boiling fractions had again been redistilled, very slowly. The procedure was to start with the lowest boiling, and separate it into perhaps three sub-fractions and an undistilled residue, with the heating of the bath and column carefully controlled by means of rheostats and ammeters, and the vapor temperature accurately noted with a thermocouple. The next higher boiling fraction would then be added, after lowering the bath and column temperatures to those at which the first fraction had begun to distil. By carefully increasing the heat, and noting the temperature of the vapors when distillation commenced, the distillate would be allowed to run into the sub-fraction already obtained which had passed over under similar conditions, commencing a new sub-fraction only when the temperature exceeded that of the highest one already obtained. In this way it was hoped that substances with boiling points differing by only a few degrees from each other would gradually become concentrated into the same fraction.

The redistillation of the entire 13 fractions in this way resulted in a total of but 11 fractions. By far the greatest bulk of the material was contained in fractions 6, 7 and 8.

Tests of the two lowest boiling active fractions, containing acetamide were now carried out, adding fractions 6 and 10 in amounts of 1.0 mgm. and 0.5 mgm. respectively to the control. These two seemed to be the most effective in supplementing the effect of the low-boiling material.

The results are shown in table 4.

This result, repeatedly confirmed and amplified, gave evidence of a substance exerting its effect over a comparatively narrow range of concentration, and either without effect or inhibitory below and above its active zone.

The combined weight of fractions 1 and 2 was 0.68 gram. About half of the material was crystalline (acetamide?). Hydrolysis of these fractions was effected by refluxing for 1 hour with 25 cc. of about 1.5 N  $\text{H}_2\text{SO}_4$ . The solution was diluted somewhat, and an excess of cold, saturated  $\text{Ba}(\text{OH})_2$  added, permitting the  $\text{NH}_3$  to be distilled out in vacuo. The excess Ba was then accurately removed with dilute  $\text{H}_2\text{SO}_4$ , and the water-clear solution evaporated to dryness in vacuo. The resulting white granu-

TABLE 4

*Effect of low-boiling material on controls supplemented by middle and high fractions*

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control with fractions 6 and 10 + fraction 1, 1.0 mgm.	1.10
2	Control with fractions 6 and 10 + fraction 1, 0.1 mgm.	1.84
3	Control with fractions 6 and 10 + fraction 1, 0.01 mgm.	2 29
4	Control with fractions 6 and 10 + fraction 2, 1.0 mgm.	1.22
5	Control with fractions 6 and 10 + fraction 2, 0.1 mgm.	2 40
6	Control with fractions 6 and 10 + fraction 2, 0.01 mgm.	2 04

Controls without fraction 1 or 2, but containing 6 and 10 produced regularly about 1.0 mgm. bacterial N.

lar material was recrystallized three times from hot alcohol and in this way were finally obtained about 10 mgm. of white crystals.

Even assuming this material to be approximately pure, too little was available for analysis with the available facilities. Fortunately it was possible to identify it another way almost as soon as obtained.

Knight (personal communication) had recently shown that his "staphylococcus vitamine" was a mixture of nicotinic acid and vitamin B<sub>1</sub>. Both these substances were at once tried in various combinations with our distillate fraction, and on the same day that the above crystals were obtained it became evident that nicotinic acid, when substituted for the low-boiling material, in

TABLE 5

*Comparison of the effects of the crystals from liver and nicotinic acid*

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control with fractions 6 and 10 + crystals, 1.0 mgm.	1 10
2	Control with fractions 6 and 10 + crystals, 0.1 mgm.	1 16
3	Control with fractions 6 and 10 + crystals, 0.01 mgm.	2 12
4	Control with fractions 6 and 10 + crystals, 0.001 mgm.	1 80
5	Control with fractions 6 and 10 + crystals, 0.0001 mgm.	1 10
6	Control with fractions 6 and 10 + nicotinic acid, 1.0 mgm.	1 12
7	Control with fractions 6 and 10 + nicotinic acid, 0.1 mgm.	1 32
8	Control with fractions 6 and 10 + nicotinic acid, 0.01 mgm.	2 92
9	Control with fractions 6 and 10 + nicotinic acid, 0.001 mgm.	1 54
10	Control with fractions 6 and 10 + nicotinic acid, 0.0001 mgm.	1 10
11	Control with fractions 6 and 10	0 92

TABLE 6

*Further comparison of effects produced by crystals from liver, nicotinic acid and its amide*

(Each tube contains 1.0 mgm. fraction 10 in the control)

SUBSTANCE	N		
	Crystals	Nicotinic acid	Nicotinamide
Control.....			0 34
mgm.	mgm.	mgm.	mgm.
0.0001	0.66	0 66	
0 00025	0.86	0.86	
0 0005	1 07	1.11	
0.001	1 51	1 51	
0.002	1.54	1.79	
0.003	1 89	1.84	
0.004	2 15	2 02	
0 005	2.20	2.18	
0.01	2.20	2 29	0 84
0.02	2.04	1 96	
0.025	1.62	1.68	(0.03 mgm.) 1.34
0.05	1.54	1 45	1 63
0.1	1.37	1 38	2 24
0.5			1 88
1.0			1.56

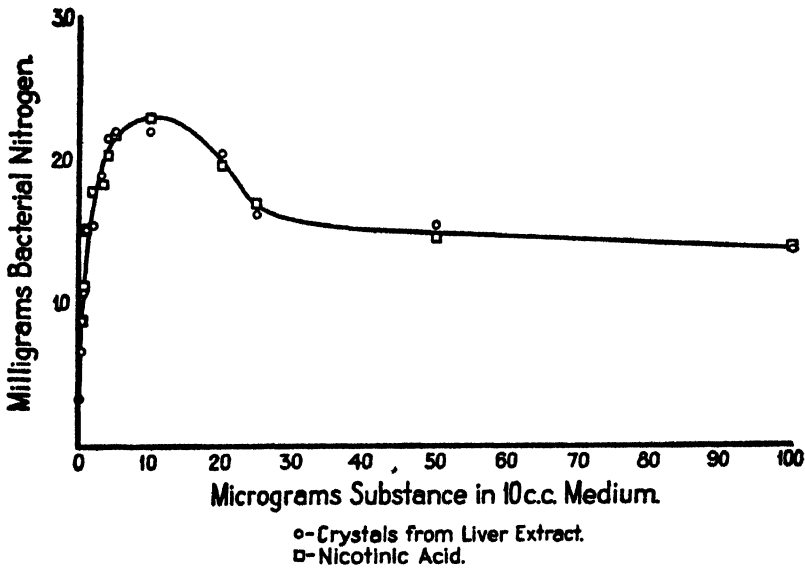


FIG. 1

TABLE 7

*The effect of crystals from liver, nicotinic acid and its amide in the absence of fractions 8 and 10*

SUBSTANCE	BACTERIAL NITROGEN		
	Crystals	Nicotinic acid	Nicotinamide
Control .....	0.10		
mgm.	mgm.	mgm.	mgm.
0.0001	0.28	0.27	
0.00025	0.40	0.40	0.13
0.0005	0.64	0.56	0.23
0.001	0.91	0.98	0.48
0.0025	0.89	0.86	0.45
0.005	0.30	0.08	0.80
0.01	0.17	0.08	1.14
0.025	0.22	0.08	1.33
0.05			0.87
0.1			0.23
0.25			0.28

the presence of fractions 6 and 10, showed the same property of growth-acceleration when the amount used was neither too great nor too little. A comparison of the solubilities and crystal form showed close similarity, and a mixed melting point made the identity practically certain. The crystals from the liver, a mixture of these with nicotinic acid, and the latter alone, melted at 234.0, 234.4 and 235.0 (uncorrected).

Growth tests set up with the crystals and nicotinic acid in parallel, shown in table 5 give equally convincing biological proof of their identity.

In table 6 and figure 1 are shown the results of a further comparison of the crystals from liver and nicotinic acid through the zone of maximum effect. Results obtained with nicotinamide, the form in which the substance may well occur in the original tissue extract, are also included in the table.

The part played by fractions 6 and 10, which have been included in the control media of the last three tables, is shown in table 7.

#### DISCUSSION

There can be no reasonable doubt that the 10 mgm. of crystalline material isolated from liver extract by the procedure outlined is nicotinic acid, and that this substance accounts for a part of the growth-promoting activity of tissue extracts for the diphtheria bacillus. That additional substances play a part of equal or greater importance, is also clear.

The effect of the nicotinic acid is peculiar in that there is evidently a well defined optimal concentration, below which, of course, no effect is exerted, and above which the effect is greatly diminished. The concentration necessary to produce maximal growth is evidently a function of the concentration of one or more other substances. For example, in the absence of "fraction 10," the heaviest growth takes place with a concentration of about 0.1  $\gamma$  per cubic centimeter of medium, while in the presence of this fraction, about ten times that concentration must be supplied. Curiously, too, the amide of nicotinic acid appears to be only about one-tenth as effective, weight for weight,

as the acid, itself, although the general type of growth curve produced is the same.

The discovery by Knight that nicotinic acid is required by the staphylococcus, taken together with the findings here presented for the diphtheria bacillus, increase the probability that a general group of substances will eventually become recognized as being the components of meat or tissue extracts so essential to the growth of many of the bacteria pathogenic for man and animals. Certain of the more fastidious organisms may require several, others only one or two. Knight finds that vitamin B<sub>1</sub> is required by the staphylococcus in addition to nicotinic acid, and other workers have found it essential for quite different types of organisms. So far, it has not been possible to demonstrate an effect of this compound on the diphtheria bacillus.

That several different extractive substances should be required by the diphtheria bacillus is not surprising. The work on amino-acid requirements reported in the earlier papers of this series suggests that the organism is definitely limited in its synthetic capabilities. There is no reason to assume that this limitation applies only to amino acids; as our general knowledge of various enzyme systems increases, to select but a single example, it becomes more and more evident that many of these involve a complex interaction of several different substances. The fact that nicotinamide takes part in at least two such systems has recently become known, due to the work of Warburg et al. (1935) and of Euler et al. (1935) and their collaborators. It is altogether probable that its requirement by the diphtheria bacillus is in connection with some such enzyme-co-enzyme mechanism, and that the organism, being unable to synthesize the pyridine nucleus, must obtain it, preformed, from its substrate.

That the greater part of the remaining growth-promoting effect of tissue extract on the diphtheria bacillus will be found due to comparatively simple substances is definitely indicated by the fact that they can be distilled without decomposition after esterification and acetylation, and will withstand acid hydrolysis. The writer hopes to be able to report later on the results of work now being carried on with these remaining factors.

## CONCLUSIONS

1. The method of Cherbuliez, Plattner and Ariel for preparation and distillation of the acetylated esters of amino acids has been applied to a study of tissue extraction required by the diphtheria bacillus for growth.

2. Distillates so prepared from a liver-extract concentrate contain the greater part of the growth-promoting activity of the extract.

3. One of the active substances has been isolated in a quantity of about 10 mgm. from 300 kgm. of liver and identified as nicotinic acid. Its maximum effect appears to be exerted at a concentration of about 1 microgram per cubic centimeter of medium but varying somewhat with the composition of the control medium. Nicotinamide is effected in approximately ten times the concentration of the free acid.

4. One or more additional active substances are also present in the distillate and are now being investigated.

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# REDUCTIVE PROCESSES OF CLOSTRIDIUM BUTYLICUM AND THE MECHANISM OF FORMATION OF ISOPROPYL ALCOHOL<sup>1</sup>

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Isopropyl alcohol as a fermentation product of certain butyl alcohol-producing bacteria was first demonstrated by Pringsheim (1906). Folpmers (1920) found it among the products of *Granulobacter butylicum* (Beijerinck) and Van der Lek (1930) reported it as a characteristic product of the same species (which he called *Clostridium butylicum* (Beijerinck, Donker). Langlykke, Peterson and McCoy (1935) showed that different strains of this organism vary markedly in the relative production of isopropyl alcohol and acetone.

Although isopropyl alcohol has long been recognized as a fermentation product, no direct evidence as to the manner of its formation has been presented. It is generally assumed to arise through the hydrogenation of acetone (Van der Lek (1930), Kluyver (1935), and Osburn (1935)). Such reductive processes are characteristic of the butyl alcohol bacteria. In the normal fermentation of carbohydrates butyric acid is first formed and then reduced to butyl alcohol. Several workers (Reilly et al. (1920), Speakman (1920), Blanchard and MacDonald (1935)) have shown that added propionic acid and aldehyde are also reduced to the corresponding alcohol.

<sup>1</sup> Supported in part by a grant from the Special Research Fund of the Graduate School.

## EXPERIMENTAL

*Cultures*

The cultures used, *Clostridium butylicum* strains 21 and 46,<sup>2</sup> have been described by Langlykke, Peterson and McCoy (1935). In addition to butyl and ethyl alcohols both produce acetone and isopropyl alcohol. Strain 21 produces little acetone while strain 46 produces both acetone and isopropyl alcohol in appreciable amounts.

*Medium*

It was desirable to employ a medium containing as little extraneous organic matter as possible. After a number of trials a medium composed of 0.07 per cent dibasic ammonium phosphate, 0.5 per cent peptone, 0.1 per cent asparagine, about 3 per cent glucose and tap water was selected. Neither peptone nor asparagine was adequate as the sole source of organic nitrogen.

In order to obtain complete fermentation, ingress of air had to be avoided. If this was not done, an incomplete, acid fermentation resulted. Entrance of air was prevented by attaching a mercury seal to the flask. The gas formed in the initial acid fermentation displaced enough air to permit the reductive phase of the fermentation to proceed.

*Analytical methods*

Glucose was determined by the method of Stiles, Peterson and Fred (1926), and lactic acid by the method of Friedemann and Graesser (1933) applied to an ether extract of an aliquot of the culture.

Neutral volatile products were determined on a distillate of the culture. For this purpose an aliquot of the culture was made slightly alkaline and about 50 per cent of the liquid was distilled off and collected under carbon dioxide-free water. The distillate was analyzed for butyl and ethyl alcohols by the

<sup>2</sup> Both strains are called *C. butylicum* for the present, although Prof. Elizabeth McCoy of this laboratory believes that, because of variations in certain characteristics, the two cultures may later have to be classified as different species.

method of Johnson (1932), acetone was determined by a modification of Goodwin's method (1920), and isopropyl alcohol by the oxidation procedure previously reported (Langlykke et al. (1935)).

For the determination of volatile acids 100 cc. of the culture were concentrated by distillation to about 40 cc. and then steam distilled until 500 cc. of distillate had been collected. Butyric and acetic acids were determined on this distillate by a modification of the procedure of Virtanen and Pulkki (1928).

For the determination of 2,3-butylene glycol an aliquot of the culture was neutralized with sodium hydroxide, taken up with plaster of Paris, and extracted with ether for 48 hours. Water was added to the extract, the ether was distilled off and the aqueous solution was oxidized to acetaldehyde by acid periodate (Brockmann and Werkman (1933)). The acetaldehyde was absorbed and determined as in the lactic acid method of Friedemann and Graeser (1933). Acetylmethyl carbinol was determined directly on the culture by the distillation procedure of Langlykke and Peterson (1937).

The procedure of Fromageot and Desneulle (1935) was applied to an ether extract of the culture for the determination of pyruvic acid. Ceric ammonium sulphate in acid solution was quantitatively reduced with the oxidation of one mol of pyruvic acid to one of acetic acid and one of carbon dioxide. There was 97 per cent recovery of pyruvic acid. When lactic acid was present, a correction of 0.098 mgm. per milligram of lactic acid was necessary.

### *Products of the fermentation*

Although there have been many quantitative studies on the fermentation products of the butyl-alcohol-producing bacteria little has been done on the identification of these products. Before beginning quantitative work it was therefore thought desirable to identify the products of one of these strains.

For this purpose 14 liters of basic medium containing 100 grams of calcium carbonate were inoculated with 400 cc. of a culture of strain 21 in 6 per cent corn mash. When fermentation was complete the culture was neutralized with sodium hydroxide

and about 30 per cent of the culture was distilled off to recover the neutral volatile products. These were then concentrated by repeated distillation, the concentrated material dehydrated with anhydrous potassium carbonate and fractionated, and the various fractions studied by the preparation of suitable derivatives.

By determination of melting points and mixed melting points with derivatives of authentic compounds it was established that n-butyl alcohol, isopropyl alcohol and acetone were produced. The 3,5-dinitrobenzoate prepared from the butyl alcohol fraction melted at 63° to 64°C. (authentic m. p., 63°C.), and the p-nitrophenylhydrazone from the acetone fraction at 149°C. (authentic, 149° to 150°C.). Isopropyl alcohol was characterized by oxidizing to acetone and preparing the dibenzalacetone (m.p., 111° to 112°C.; authentic, 110° to 111°C.) by the reaction with benzaldehyde. Direct evidence of the presence of ethyl alcohol could not be obtained. If this compound is produced, it is in such small amounts that a separation could not be effected. The evidence for the production of ethyl alcohol is admittedly weak and lies in the fact that a small value for ethyl alcohol is always obtained in the analysis by the method of Johnson (1932).

Butylene glycol could not be demonstrated in the culture residue after evaporation. Several other fermentations were examined for acetylmethyl carbinol and for 2,3-butylene glycol by the method of Lemoigne as modified by Kluyver et al. (1925), but the results were uniformly negative.

#### *Reduction of acetone*

To determine whether acetone when added to the culture would be reduced, a number of experiments were carried out. A series of 750-cc. Erlenmeyer flasks containing the basic medium were sterilized at 15 pounds pressure for 40 minutes. Sufficient sterile glucose solution was added to bring the glucose content to 3 per cent, and then varying quantities of a sterile acetone solution were added. In each case the total volume was adjusted to 500 cc. by addition of sterile water. A two per cent inoculum of a corn mash culture of the organism was then added

to each flask except the controls, the mercury seals were applied, and the flasks allowed to incubate at 37°C. When fermentation was complete, as evidenced by the cessation of gassing, the flasks were analyzed for products.

Data for strain 21 are presented in table 1. They indicate that the added acetone was almost completely converted to isopropyl alcohol, and that, as the quantity of added acetone was increased, there was an increased formation of isopropyl alcohol from the carbohydrates. This is due in part to the better fermentation of the carbohydrate when acetone was added, but probably another factor is involved.

TABLE 1

*Reduction of added acetone in association with the fermentation of glucose (strain 21)*

	EXPERI- MENT 1	EXPERI- MENT 2	EXPERI- MENT 3	EXPERI- MENT 4
	mM	mM	mM	mM
Glucose fermented, per liter . . . . .	122 7	124 5	129 5	136 1
Acetone added:				
Per liter . . . . .	0 0	11.3	22 6	33 9
Per 100 mM. glucose fermented . . . .	0 0	9.1	17 5	24 9
Products (based on 100 mM. of glucose fermented):				
Butyric acid . . . . .	9 85	8 70	8 25	6 70
Acetic acid . . . . .	9 45	8 90	8 65	8 10
Butyl alcohol . . . . .	50 5	57 5	54 6	55 8
Ethyl alcohol . . . . .	3.3	2 6	2 2	1 5
Acetone . . . . .	1.5	2.7	3 6	3 4
Isopropyl alcohol . . . . .	16 1	26 9	39 2	51.0

It will be noted that as added acetone was increased, the ratio of the sum of isopropyl alcohol and acetone formed from the carbohydrate to the sum of butyl alcohol and butyric acid increased (from 0.29 in experiment 1 to 0.41 in experiment 4). In other words, production of three-carbon compounds increases at the expense of four-carbon compounds in the presence of the hydrogen acceptor, acetone.

In the case of strain 46 the cultures containing added acetone had stopped gassing after five days while in those without added acetone gas was still forming at this time. Therefore the blank

fermentations were incubated for eight days. In spite of these differences in fermentation periods the results (table 2) show the general tendencies pointed out for strain 21.

The great differences in the amounts of glucose fermented prevent direct comparison, but if experiment 4 and the control, which do not differ greatly in this respect, are compared it will be seen that the total production of acetone and isopropyl alcohol from the substrate is somewhat greater when acetone is added, though apparently acetone does not function so efficiently as a hydrogen acceptor for this strain as it does for strain 21.

TABLE 2

*Reduction of added acetone in association with the fermentation of glucose (strain 46)*

	EXPERI- MENT 1	EXPERI- MENT 2	EXPERI- MENT 3	EXPERI- MENT 4
	mM	mM	mM	mM
Glucose fermented, per liter. . . . .	69.2	29.3	45.8	74.6
Acetone added:				
Per liter. . . . .	0.0	13.1	26.3	39.5
Per 100 mM. glucose fermented . . . . .	0.0	44.6	57.4	53.0
Products (based on 100 mM. of glucose fermented):				
Butyl alcohol . . . . .	39.3	35.1	29.9	40.1
Ethyl alcohol. . . . .	1.9	2.0	1.7	0.9
Acetone. . . . .	5.6	15.4	30.4	26.8
Isopropyl alcohol. . . . .	18.8	46.7	54.5	57.6

### *Fermentation of pyruvic acid*

Since the organisms studied could reduce the carbonyl group in acetone, experiments were conducted to determine whether this function was general or specific. For instance, if they could reduce pyruvic acid to lactic, the position of pyruvic acid as an intermediate in the fermentation mechanism might be doubtful.

A series of 750-cc. Erlenmeyer flasks containing the basic medium were sterilized at 15 pounds pressure for 40 minutes. Pyruvic acid which had been twice redistilled under vacuum from the Eastman technical grade was added aseptically to sterile water to make a solution of convenient concentration. Glucose solution, pyruvic acid solution and sterile water were

then added aseptically to each flask to give a final volume of 500 cc. and varying glucose and pyruvic acid concentrations as shown in table 3. An excess of calcium carbonate was added to each flask which was then inoculated with 2 per cent of a corn mash culture of strain 21.

After incubation for five days at 37°C. the cultures were analyzed with results shown in table 3. The data indicate a slight increase in the apparent lactic acid production, but the major portion of the added pyruvic acid is accounted for in the normal products of the fermentation. This does not demonstrate,

TABLE 3  
*Dissimilation of pyruvic acid added to a glucose fermentation (strain 21)*

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
	mM	mM	mM
Glucose fermented, per liter. . . . .	179.8	149.6	84.4
Pyruvic acid fermented:			
Per liter. . . . .	0.0	47.7	125.4
Per 100 mM. of glucose fermented . . . . .	0.0	31.9	148.7
Products (based on 100 mM. of glucose fermented):			
Butyric acid. . . . .	8.8	7.8	32.2
Acetic acid. . . . .	17.1	17.7	77.6
Lactic acid. . . . .	1.1	1.6	7.1
Butyl alcohol. . . . .	44.6	50.6	41.7
Ethyl alcohol. . . . .	2.6	3.5	6.2
Acetone. . . . .	1.6	2.5	4.0
Isopropyl alcohol. . . . .	15.6	30.6	30.4

however, that pyruvic acid is not directly reduced to lactic acid since this process may occur followed by metabolism of the lactic acid so formed. (Lactic acid, in the presence of glucose, is decomposed by strain 21.)

Like acetone, pyruvic acid acts as a hydrogen acceptor, though this compound is not simply reduced. The ratio of the sum of isopropyl alcohol and acetone to the sum of butyric acid and butyl alcohol increased from 0.33 to 0.57 in the second experiment where 47.7 millimols of pyruvic acid were fermented. The apparent anomaly observed in experiment 3, where the ratio



dropped to 0.47, is due to the high production of acids. If these acids, which are also hydrogen acceptors, are not metabolized, more hydrogen is available for the other normal functions of the cell.

*Reduction of acetylmethyl carbinol*

As was previously pointed out, neither acetylmethyl carbinol nor its reduction product, 2,3-butylene glycol, appears among the products of the fermentation by *C. butylicum*. Hence, added acetylmethyl carbinol, if directly reduced, might serve for study

TABLE 4

*Reduction of added acetylmethyl carbinol in a glucose fermentation (strain 21)*

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
	mM	mM	mM
Glucose fermented, per liter. . . . .	95 8	69 1	53.8
Acetylmethyl carbinol fermented:			
Per liter. . . . .	0 0	11.7	35.6
Per 100 mM. glucose fermented. . . . .	0 0	16.9	66.2
Products (based on 100 mM. of glucose fermented):			
Butyric acid. . . . .	9 7	14.6	19.7
Acetic acid. . . . .	21 1	29.5	26.0
Butyl alcohol. . . . .	47.0	32.9	22.8
Ethyl alcohol. . . . .	2.9	4 3	14.9
Acetone. . . . .	2.0	2 3	3.0
Isopropyl alcohol. . . . .	11 7	12.5	15.8
2,3-Butylene glycol. . . . .	0.0	17.5	66.0

of the effect of a hydrogen acceptor on the course of the fermentation, without the objections attending the use of a hydrogen acceptor which also performs other functions.

Essentially the same procedure as in the preceding experiments was followed. The acetylmethyl carbinol solution was sterilized by filtration through a Berkefeld filter. The flasks were inoculated with strain 21 and were incubated at 37°C. for four days; gassing had then ceased. The cultures were analyzed with results reported in table 4.

The figure for acetylmethyl carbinol utilized represents the

difference between that added and the amount remaining after fermentation. Actually the reduction was in each case approximately 95 per cent complete. Quantitatively, the yield of butylene glycol was 103.6 per cent in the case of experiment 2 and 99.7 per cent in experiment 3, based on the acetylmethyl carbinol utilized.

Although in each instance poor fermentations of the glucose were obtained with a high proportion of unconverted acetic acid, still the effect of the presence of a hydrogen acceptor may be noted. The ratio of three-carbon to four-carbon products increased from 0.24 in experiment 1 containing no additions to 0.31 in experiment 2 and 0.43 in experiment 3.

#### DISCUSSION

The behavior of *C. butylicum* fermentations when a hydrogen acceptor is present may be explained on the assumption that both acetone and butyric acid arise from a common precursor, acetoacetic acid. Johnson, Peterson and Fred (1933), have shown that the related organism, *Clostridium acetobutylicum*, produces a carboxylase which readily causes the formation of acetone from acetoacetic acid, and it seems reasonable to suppose that the latter compound may also be hydrogenated to butyric acid provided that the hydrogen for this reduction is available. Presence of an acceptor competing for the available hydrogen for reduction would disturb the balanced reactions and favor the decarboxylation reaction by which acetone is produced over the reductive process through which butyric acid arises.

It is true that the behavior noted is not proof that the three- and four-carbon products come from a common precursor. They may arise through separate reaction chains which would be similarly affected by the paucity of hydrogen because of the presence of a hydrogen acceptor. Thus, butyl alcohol may arise by condensation of two molecules of acetaldehyde to acetaldol, followed by rearrangement to butyric acid, and hydrogenation to butyl alcohol. It is not probable that acetaldol functions as an intermediate, as it has been shown by Johnson, Peterson and Fred (1933) and also by Blanchard and McDonald (1935)

that aldol is extremely toxic to the related organism, *C. acetobutylicum*. Bernhauer and Kurschner (1935) agree that aldol does not figure in the fermentation mechanism of *C. acetobutylicum*. The assumption that acetoacetic acid is the common precursor of acetone and butyric acid, and subsequently of isopropyl and butyl alcohols, merely offers the most convenient explanation of the phenomena observed.

The effect of hydrogen acceptors on the course of the fermentation effected by *Lactobacillus lycopersici* has been studied by Nelson and Werkman (1936). In agreement with the results reported in this investigation these authors found that acetaldehyde and acetylmethyl carbinol were readily hydrogenated by their organism, and that the presence of these hydrogen acceptors caused an increase in oxidized products at the expense of reduced products formed from the carbohydrate.

#### SUMMARY

1. Acetone and acetylmethyl carbinol added to glucose fermentations were reduced by *Clostridium butylicum* to the corresponding alcohols, isopropyl alcohol, and 2,3-butylene glycol.

2. The quantity of acetone reduced varied with different strains of the organism.

3. Added pyruvic acid was fermented to the same products as are formed from glucose.

4. Addition of acetone, pyruvic acid or acetylmethyl carbinol favors the production of isopropyl alcohol and acetone from the carbohydrate at the expense of butyl alcohol and butyric acid production.

5. A proposed explanation of the effect of hydrogen acceptors is that both three- and four-carbon compounds may arise from a common precursor, acetoacetic acid.

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## A NEW CULTURE MEDIUM FOR RHIZOBIA<sup>1</sup>

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Calcium exists in by far the largest amount among the exchangeable cations in a good soil, while available, or possibly exchangeable phosphorus is small in comparison. In the ordinary medium used for the growing of Rhizobia (Ashby, 1907) quite the reverse is true; namely, it contains a liberal supply of soluble phosphorus and a limited supply of soluble calcium. This reversal by the laboratory culture medium of the ratio of exchangeable calcium to phosphorus common in the soil may help to explain scant and slow growths, variant forms, and the dying out of cultures, commonly encountered in laboratory practices.

Calcium adsorbed on the colloidal fraction of the soil was delivered to the bacteria in larger amounts than was possible from a laboratory medium with the same level of calcium as a carbonate. The adsorbed calcium proved most effective in promoting the growth of Rhizobia (McCalla, 1937), especially of the slow-growing varieties such as *Rhizobium japonicum*. This pointed to the need in the laboratory medium for a high level of calcium in a more readily usable but not highly ionized form.

Calcium gluconate, which furnishes a soluble but not highly ionized compound; whose anion is structurally analogous to sugar; and which lowers the surface tension of the medium by accumulating in the surface next to the bacterial cell, suggested itself as a means of providing such a supply of this nutrient.

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Substituting such a soluble compound for calcium carbonate was considered advisable since it would give a clear medium with a high buffer capacity, would stimulate slow growers, and possibly would serve in lessening contamination.

Accordingly the following medium for *Rhizobia* was tested:

Sodium chloride.....	0.2 gram
Magnesium sulphate.....	0.2 gram
Sucrose.....	10.0 grams
Calcium gluconate.....	1.5 grams
Dissolve the above in distilled water.....	940.0 ml.
Sauer kraut juice.....	20.0 ml.
Hardwood ash extract.....	30.0 ml.
(Boil 4 grams ash in 60 ml. water and filter after standing)	
Dipotassium phosphate N/10.....	10.0 ml.
For solid medium add agar-agar .....	15.0 grams
Adjust to pH 7.5 by means of potassium hydroxide.	

As an "accessory growth substance," sauer-kraut juice was substituted for the yeast extract formerly used. Since yeast itself probably demands "accessory growth substances" of vegetable origin, it would seem logical to go directly to their source rather than to obtain them indirectly through the yeast. Trials of many unfermented vegetable extracts showed these to be superior to the yeast extract but not as potent as the sauer-kraut juice in speeding the growth of the microorganisms. The wood-ash extract was used in order to avoid the omission of some essential but less common nutrient minerals. It serves also to contribute some potassium.

This medium has been used in the laboratory with decidedly improved results over the Ashby (1907) medium. This improvement was noted especially in the increased growth rate of the so-called "slow growers." No abnormal or variant forms have appeared to date in a large number of cultures produced. Contamination has also been less than during the use of other media. The behavior of the *Rhizobia* on this medium, as judged by rates of multiplication and retention of common characters, suggests that the medium is more nearly adapted to the needs of these microorganisms and will serve more effectively for their growth in the laboratory than the media commonly used in the past.

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# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## CENTRAL PENNSYLVANIA BRANCH

BUCKNELL UNIVERSITY, LEWISBURG, PENNSYLVANIA, MAY 15, 1937

**SYPHILIS FROM THE STANDPOINT OF THE CLINICIAN.** *Peter P. Mayock*, Chief of State Genito-Urinary Clinic, Wilkes-Barre, Pennsylvania.

**SOCIAL WORK AND SYPHILIS.** *Nellie Loftus*, Chief Nurse and Social Worker, State Clinic, Wilkes-Barre, Pa.

**STENOSING PROCTITIS, OR THE SIXTH VENEREAL DISEASE.** *G. N. Fleugel*, State Genito-Urinary Clinic, Wilkes-Barre, Pennsylvania.

**MODERN PROBLEMS IN THE EXPERIMENTAL STUDY OF SYPHILIS.** *Harry Eagle*, United States Public Health Service, Washington, D. C. and the Johns Hopkins Medical School, Baltimore, Maryland.

Contrary to the accepted teaching of 25 years, it is questionable whether the *Spirocheta pallida* has ever been successfully cultivated on artificial media. Such regular cultivation of a truly pathogenic *Spirocheta pallida* is perhaps the most pressing problem in syphilis research.

In the serodiagnosis of syphilis, it is important that serologists devise new, more sensitive and more reliable modifications of the laboratory tests; but it is even more important that laboratory technicians learn to use properly those technics which are already available.

The nature of the reactive substance in syphilitic serum has been a long-standing mystery. It seems a plausible working hypothesis, consistent with the known facts, that it is simply an antibody to the *Spirocheta pallida*. That this antibody reacts with a non-specific antigen (e.g., beef heart lipoid) may simply be due to the presence in the spirochete and in mammalian tissue of a serologically related antigenic factor, a cross-reaction for which there are numerous analogies.

The nature of the "immunity" (i.e., resistance to re-infection) engendered by syphilitic infection is as yet wholly obscure. The elucidation both of the biologic properties of the *Spirocheta pallida*, and of the nature of the antibody response in the infected individual, have been seriously handicapped by our inability to grow the organism *in vitro*.

With respect to the chemotherapy of syphilis, although the arsphenamines constitute a therapeutic agent of proved value, the mechanism of their action is as yet obscure. The elucidation of this problem may well provide a clue to the development of more effective, more rapid or safer methods of treatment.

**TUBERCULOUS TRACHEOBRONCHITIS. PRELIMINARY REPORT OF ITS TREATMENT BY ELECTROCOAGULATION.** *F.*

W. Davison, Department of Otolaryngology and Bronchoscopy, The George F. Geisinger Memorial Hospital, Danville, Penn.

The pathology of tuberculous tracheobronchitis has been well described by Dr. Leo Eloesser. Accurate diagnosis of these lesions is not possible without bronchoscopic examination and it is only recently that the bronchoscope has been used as a means of diagnosis in tuberculous patients. During the past nine months we have had the opportunity of observing and treating three patients with tuberculous, tracheal and bronchial lesions. While the number of patients treated is small, the results have been so consistently satisfactory that I think it is worth while to make a preliminary report with the hope that others will give this method a trial.

I have been using an electro-coagulation current of low intensity, not with the idea of destroying all the tuberculous tissue present, but with the idea of inducing an acute inflammatory reaction locally, which results in increased blood supply and in hastening production of fibrous tissue.

No harmful effects were noted as a result of bronchoscopic examination and in each case the tracheo-bronchial lesions showed a consistent tendency to heal under the influence of treatment with a mild electro-coagulation current.

**THE RELATION OF CATALASE ACTIVITY TO THE MICROFLORA OF CURED AND FERMENTING TOBACCO.** *J. J. Reid, D. E. Haley, D. W. McKinstry and J. D. Surmatis*, Division of Bacteriology and Department of Agricultural

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An extensive flora was found on the leaves of cured Pennsylvania cigar-leaf tobacco. Spores of members of the genus *Bacillus*, cocci, and fungi of the genera *Aspergillus*, *Penicillium* and *Rhizopus* comprised the major portion of the microflora present. Tobacco of poor quality showed fewer bacteria than tobacco of good quality, although in some cases the numbers of fungi present were greater than in the case of the better grades of tobacco.

Catalase activity of the cured tobacco was found to be directly related to the numbers of microorganisms present. High bacterial counts were invariably associated with marked catalase activity and low counts with little or no activity.

During the fermentation process a marked increase in bacterial numbers was noted and this in every instance was accompanied by a relative increase in catalase activity. Vegetative cells of the genus *Bacillus*, occurring in chains of from 2 to 8 organisms, were observed.

Any treatment of tobacco attempted which was found to inhibit the reproduction of microorganisms, likewise prevented an increase in catalase activity. Treatments, on the other hand, that resulted in an increase in bacterial numbers, resulted in an increase in catalase activity.

Normal catalase activity was restored to tobacco rendered inactive by heat by means of inoculation with pure cultures of bacilli isolated from cured and fermenting tobacco.

# THE APPLICATION OF SINTERED (FRITTED) GLASS FILTERS TO BACTERIOLOGICAL WORK

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Glass disks, prepared by sintering finely pulverized glass in a suitable mold (Bruce and Bent, 1931; Kirk, Craig and Rosenfels, 1934; Shatenshtein, 1929) have found applications in the fields of chemistry (Prausnitz, 1924, 1929 and 1932), microchemistry (Kirk, 1933) and pharmacy (Prausnitz, 1930). As far as is known, there is no account in the literature of their employment in bacteriological work, except for the statement (Kirk, 1937) that they have been used satisfactorily for the sterilization of Tyrode's solution, peptic digests of protein and peptone and other materials of a similar nature.

Very often in bacteriological work it is necessary to filter a small amount of material or to have a filter which is chemically clean as well as sterile. In immunological work it is imperative that filters be free from all foreign antigens. Sintered glass filters present these advantages, if they can be depended upon to withhold bacteria.

The filters<sup>1</sup> used in our work are of Jena glass of the type shown in figure 1. The sintered glass disk, 30 mm. in diameter, is of "5 auf 3" porosity. The capacity of the funnel above the sintered glass disk is 30 cc. The funnel is fitted into a test tube with a side arm by means of a rubber stopper. After plugging the side arm with cotton, the assembly is autoclaved at 120°C. for 30 minutes. A pressure of about 75 cm. of mercury is applied during the filtration process. After use, the funnels are disconnected from the test tube, placed in distilled water and boiled

<sup>1</sup> Obtained from Fish-Schurman Corporation, 250 E. 43rd Street, New York.

for 15 minutes. The filters are then chemically cleaned by placing them in concentrated  $\text{H}_2\text{SO}_4$  to which is added a little  $\text{NaNO}_3$  and  $\text{NaClO}_4$ , heating to  $80^\circ$  to  $90^\circ\text{C}$ . and allowing the solutions to act overnight. After rinsing the filters well, about 200 cc. of distilled water were filtered, as it was found that at least 125 cc. were required to pass through the filter before all traces of acid were removed. The conventional  $\text{H}_2\text{SO}_4$ - $\text{K}_2\text{Cr}_2\text{O}_7$  cleaning solution was not used because of the objections pointed out by Laug (1934).

Broth cultures of the following organisms have been filtered: *Escherichia coli*, *Shigella dysenteriae* (Shiga), *Eberthella typhosa*,



FIG. 1

*Serratia marcescens*, *Corynebacterium diphtheriae*, *Hemophilus influenzae*, *Proteus vulgaris*, *Staphylococcus albus*, *Streptococcus hemolyticus* (Group A), *Vibrio cholerae* and *Vibrio metchnikovi*. In no case was the organism in question ever encountered in the filtrates. The volume of culture filtered at any one time varied from 3 to 50 cc. In the case of *Proteus vulgaris* the filtration process was carried out over a period of 5 hours. If the culture was heavy, filtration was likely to be very slow. In most cases sediments were avoided or the culture was centrifugalized for a short period and the supernatant employed for filtration.

Having successfully withheld the organisms enumerated above in every instance, the filters were then employed for various other

purposes. The most frequent use has been for the preparation of sterile solutions of carbohydrates to be added to culture media. Solutions of cellobiose, glucose, glycerol, inositol, lactose, maltose,  $\alpha$ -methyl glucoside, rhamnose, sucrose, salicin and trehalose have been filtered through the "3 G 5 auf 3" filters and have been found to be sterile in every instance. The filters were, likewise, found satisfactory for filtration of staphylococcus bacteriophage and dysentery bacteriophage, the group specific carbohydrate (Lancefield, 1933) and labile antigen (Mudd *et al.*, 1937) of Group A  $\beta$ -hemolytic streptococci, and tetanus toxin. Small amounts of rabbit immune sera have also been rendered sterile by filtration through the filters. They are especially advantageous when only small amounts of material are available. In the case of the glass filters only 0.5 cc. is lost in the filter and in manipulation, whereas in the medium sized Berkefeld filters the loss is about 4.5 cc.

The candles are still in use, and, after being used some twenty times, are filtering as rapidly as when new, i.e., 50 cc. in about 28 minutes.

Like the Berkefeld filters (Mudd, 1928) they carry a negative electrical charge; decolorizing the first portions of dilute solutions of gentian violet, but allowing a negatively charged dye, such as Congo red, to pass through.

#### SUMMARY

We have found Jena glass filters of "5 auf 3" porosity dependable in withholding bacteria and highly advantageous in cases where it is necessary to work with small amounts, and where it is desirable to use a filter which is free from antigenic substances.

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# PRODUCTION OF VARIANTS OF THE COLON AND AEROGENES GROUPS IN DIFFERENT MEDIA

## I. SUCROSE MEDIUM<sup>1</sup>

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### INTRODUCTION

The investigations of Neisser (1906), Massini (1907), Kowalenko (1910), and Baerthlein (1918) did much to establish bacterial variation or dissociation. A comprehensive review of the research on this subject has been given in articles by Hadley (1927), Dulaney (1928), Lewis (1934), and Torrey and Montu (1936); and will not be repeated in this publication.

Most investigators, however, have concerned themselves with the variation in morphological and colonial characteristics, especially in regard to the smooth (S) and rough (R) types. Neisser (1906), in his first work on variation, noted that a non-lactose-fermenting colon-like organism gave daughter colonies which fermented lactose. Burri (1910) observed this same phenomenon in regard to sucrose fermentation, when he studied an organism isolated from fermenting grass.

Many investigators have found no characteristic bio-chemical difference among their variants when the original culture was grown in media containing carbohydrates or higher alcohols, except lactose. The addition of certain chemicals to the media, however, has produced variants with new fermentation reactions. For instances, Dawson (1919) employed fat and Stearn (1923) dyes in media for the growth of *Escherichia coli*, and found that this organism developed the power to ferment sucrose. Smirnow

<sup>1</sup> The material in this communication formed a part of a thesis submitted by the senior author in 1935. Univ. of Colo. Studies, vol. 23, pp. 74-75 (1935).



(1916), on the other hand, found that phenol media caused members of the colon group to lose their power to ferment carbohydrates.

Variation occurs spontaneously under ordinary conditions of cultivation and also with environmental stimuli such as the presence of sugars, dyes, and changes in temperature. We have chosen the presence of sucrose in the medium as the environmental stimulus in this present investigation. The chief object was to study the characteristics of the variants which were produced when separate individual colonies from the mother culture were successively transplanted in sucrose broth for 15 times.

#### PROCEDURE AND MATERIAL

The cultures used in this investigation were obtained from the laboratory collection which has been kept in the department for over ten years. During this time, fresh slants were made every two or three months, and the cultural and morphological characteristics were checked from one to four times each year.

The sucrose medium was prepared by adding 8 grams of Bacto-nutrient broth and 5 grams of sucrose to 1 liter of distilled water. The pH was adjusted to 7.0 to 7.1, and the medium was sterilized at 15 pounds pressure for 15 minutes. The eosin-methylene-blue medium was made from the dehydrated preparation prepared by the Difco Laboratories according to the formula of Levine.

A group of 36 sucrose-negative organisms were chosen, 33 of these belonging to the *Escherichia* group and 3 to the *Aerobacter* group. These cultures had been purified by the streak method at least 15 times, and have given during the last 10 years negative reactions after 10 days' incubation in sucrose medium. Eosin-methylene-blue plates were streaked from the stock cultures of these organisms. After incubation, one well-isolated colony was used for restreaking on eosin-methylene-blue agar. Twenty-five colonies were selected from this later plate and each colony was separately transferred to sucrose medium contained in Durham fermentation tubes. Transfers of 10 of the cultures which showed no gas production in 7 days were made every third day to fresh tubes of sucrose medium for 15 times.

## EXPERIMENTAL RESULTS

The percentage of gas produced in the Durham tubes of each of the transfers after 7 days' incubation was carefully recorded. In most cases, it was found that all of the 25 colonies first inoculated into sucrose medium were negative to this sugar. In a few cultures, occasional colonies were found which were positive to sucrose. Sherman and Wing (1937) have just reported this same phenomenon. If an organism showed one or more positive sucrose reactions in any of the 25 sub-cultures, this organism was not used for further examination.

Out of the 36 cultures finally tested, 27 (270 transplants) remained negative to sucrose throughout the 15 times of sub-culturing in sucrose broth. Nine of the cultures showed an acquired ability to ferment sucrose in from 1 to 10 of the transplants. From the cultural reactions, the nine original organisms were named according to Bergey. There were four cultures of *Escherichia coli*; 1 of *Escherichia alba*; 2 of *Escherichia gruentali*; and 2 of *Aerobacter levans*.

In table 1 are shown the percentages of gas produced by the variants of cultures of *E. coli*. Table 2 shows the results for the cultures belonging to the species *E. alba*, *E. gruentali* and *A. levans*.

After the sucrose-positive organisms had been transplanted and grown for 15 times in sucrose medium, streaks were made of each culture. Twenty-five colonies were picked at random and placed in sucrose broth in order to determine whether or not all the sub-cultures were positive to sucrose. The majority of the tubes gave gas production, but in some cases there were a few of the sub-cultures which did not yet produce gas in sucrose broth. Throughout the experimental work, no difference was noticed as to the character of the colonies when the sucrose-positive and sucrose-negative variants were grown on plain agar and eosin-methylene-blue agar. Each variant was tested for motility, indol production, liquefaction of gelatin, for the V.P.—M.R. reaction; and for fermentation of  $\alpha$  methyl glucoside, sucrose, salicin, dulcitol, cellose, and raffinose. No cultural reaction with the exception of sucrose fermentation had been changed.

TABLE 1  
*Production of gas*  
Sucrose-positive variants

NUMBER OF TRANS- PLANT	E. COLI NO. 43										E. COLI NO. 113										E. COLI NO. 28										E. COLI NO. 224									
	Variant																																							
	1†	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7			
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
3	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
4	0	10	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
5	10*	10	0	0	0	0	0	5	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
6	20	10	0	0	0	0	0	5	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
7	40	10	0	0	0	0	0	10	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
8	35	10	0	0	0	0	0	15	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
9	30	15	0	0	0	0	0	30	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
10	25	15	0	0	0	0	15	30	30	0	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
11	30	20	10	20	25	20	35	30	30	0	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
12	85	15	15	30	25	20	40	35	35	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5				
13	90	25	20	25	20	20	35	30	30	0	10	5	5	10	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15				
14	90	25	30	40	25	15	35	35	35	0	30	30	15	15	30	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25				
15	90	20	35	50	25	15	40	35	35	0	40	30	25	30	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35				

\* Figures are in percentage gas produced in seven days.

† The numbers omitted up to 10 represent the colonies which remain negative to sucrose during the fifteen transplants.

TABLE 2  
*Production of gas*  
Sucrose-positive variants

NUM- BER OF TRANS- PLANT	A. LEVANS NO. 346								A. LEVANS NO. 422										E. ALBA NO. 13							E. GRUENTHALI NO. 184							E. GRU- ENTHALI NO. 248
	Variant								Variant																								
2†	4	6	7	8	1	2	3	4	5	6	7	8	9	10	1	2	3	5	7	1	2	5	7	8	9	1	2	5	7	8	9		
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
3	0	0	0	0	0	5	0	0	5	0	0	0	0	5	0	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0		
4	0	0	5	0	0	30	30	20	50	30	40	10	10	20	0	10	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0		
5	0	0	10	5	5	50	50	25	50	30	40	15	20		0	15	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0		
6	0	0	10	5	10	40	50	25	35	50	30	40	60	35	0	15	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0		
7	0	0	15	10	15	40	40	20	30	50	50	50	75	40	0	15	0	0	20	20	0	0	0	0	0	0	0	0	0	0	20		
8	0	0	15	15	20	60	60	20	30	40	50	50	80	35	0	20	0	0	25	20	0	0	0	0	0	0	0	0	0	0	55		
9	0	10	20	15	35	40	70	50	60	65	65	70	80	70	50	0	20	0	0	25	20	0	0	0	0	0	0	0	0	0	50		
10	10*	15	20	20	35	60	65	40	60	60	60	70	75	80	5	25	5	0	25	25	5	20	10	0	10	50	50	50	50	50	60		
11	15	15	25	15	30	70	80	60	70	70	70	75	65	70	20	30	30	20	30	30	15	25	30	0	20	50	50	50	50	50	55		
12	10	15	25	20	30	75	65	50	80	45	45	65	70	50	20	30	25	25	50	20	20	30	40	5	20	55	50	50	50	50	55		
13	10	15	20	20	35	60	50	50	60	40	45	60	60	60	25	60	30	60	70	40	20	30	30	10	25	50	50	50	50	50	60		
14	10	15	25	25	40	60	50	45	60	60	55	65	50	35	20	70	70	70	70	30	30	35	35	10	35	60	60	60	60	60	75		
15	20	25	20	30	40	75	45	55	65	40	45	50	60	40	25	80	90	70	80	40	35	35	50	10	35	75	75	75	75	75	75		

\* Figures are in percentage gas produced in seven days.

† The numbers omitted up to 10 represent the colonies which remained negative to sucrose during the fifteen transplants.

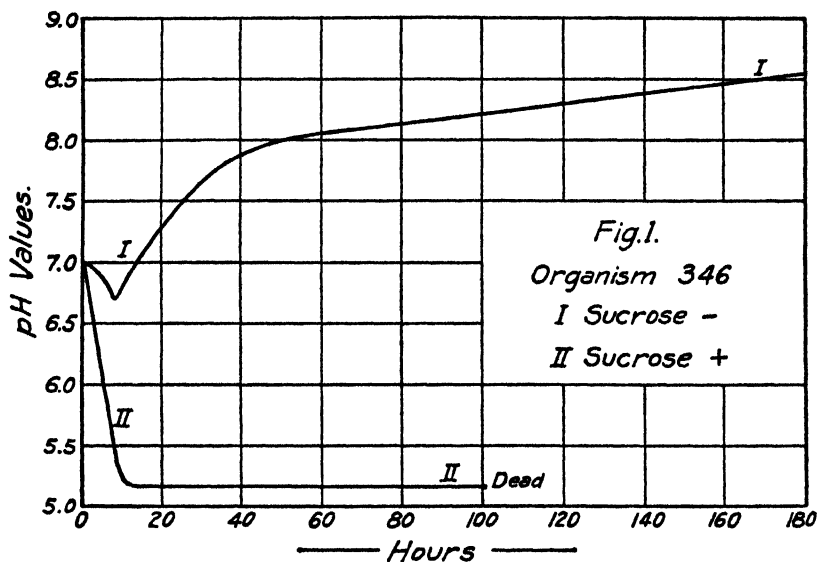


FIG. 1. CURVES SHOWING CHANGES IN pH BY AN ORIGINAL CULTURE OF *A. LEVANS* AND ONE OF ITS SUCROSE-POSITIVE VARIANTS WHEN EACH IS GROWN IN SUCROSE MEDIUM

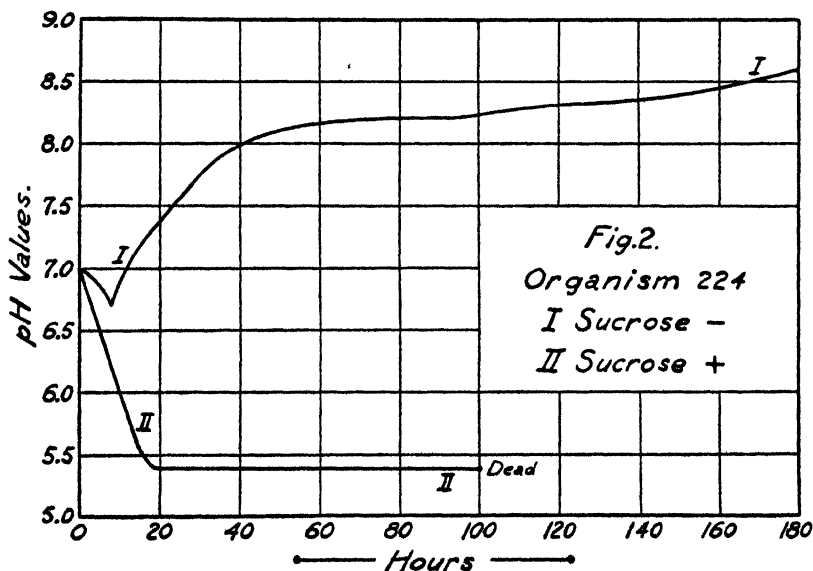


FIG. 2. CURVES SHOWING CHANGES IN pH BY AN ORIGINAL CULTURE OF *E. COLI* AND ONE OF ITS SUCROSE-POSITIVE VARIANTS WHEN EACH IS GROWN IN SUCROSE MEDIUM

The quantitative study of the progressive acid fermentation was conducted on one of the original cultures of *A. levans* and on one of its variants which developed the power to ferment sucrose. These organisms were grown in sucrose medium, and at frequent intervals during 180 hours, the pH values of the medium were determined. The curves representing the results of the change in pH are given in figure 1. Similar determinations were made for a culture of *E. coli* and one of its sucrose-positive variants. These results are shown in figure 2.

A representative number of the sucrose-positive variants were placed on agar slants for permanent cultures. All of these cultures were transferred to sucrose broth after being stored for two years at about 4°C. In every case where the organisms were still alive the reaction to sucrose was still positive.

#### DISCUSSION OF RESULTS

The production of sucrose-positive variants is easy to accomplish with some members of the *Escherichia* and *Aerobacter* groups. Of the 36 cultures tested, 9 or 25 per cent exhibited the power to produce variants which fermented sucrose. Some strains produced the variants with ease, as was the case with organism number 422. All the 10 sub-cultures of this organism began to ferment sucrose in 4 days. Other organisms acquired the ability with less ease, as may be seen from the results given by organism number 248, which produced only 1 sucrose-positive variant. Of the total of 90 transplants from the 9 organisms, 58 per cent acquired the ability to ferment sucrose.

The ability of bacteria to decompose carbohydrates with acid and gas production has been accepted by most bacteriologists as one of the most valuable means of classifying bacteria. The primary reason for making the investigation reported in this paper was to determine whether or not, in a classification like the one proposed by Bergey, the use of these fermentation reactions for the separation of species could be justified.

The results of the experiment submitted herewith seem to indicate that the fermentation of sucrose is not necessarily a constant characteristic. The acquired ability of the organisms

used in this research to ferment sucrose would, according to Bergey, place the mother colony and the variant in different classifications. The original cultures of *E. coli*—numbers 28 42, 113, and 224—were changed to *Escherichia communior*; those of *A. levans*—numbers 346 and 422—were changed into *Aerobacter hibernicum*; those of *E. gruenthali* to *Escherichia anindolica*; and that of *E. alba* to *Escherichia gastrica*.

In studying the pH curves in figures 1 and 2, it will be discovered that the sucrose-positive variants developed a great capacity to form acid from sucrose. The original cultures showed an acid production for about 7 hours to a minimum pH of about 6.7; and then there was an abrupt rise in the curves until a pH of over 8.5 was reached in 180 hours. The sucrose-positive variants from these cultures, however, showed a rapidly descending curve to a pH value from 5.2 to 5.4 in 10 to 15 hours. The production of acid in the sucrose-positive variants was so great that it caused the death of the organism in most cases.

It is not surprising that an organism fails to retain its stability when it is removed from its natural habitat and placed in an artificial environment. The results in this paper would justify the statement that the use of fermentative reactions, as obtained from sugar media, is not entirely satisfactory as a basis of the subdivision of the *Escherichia* and *Aerobacter* groups of bacteria. Sherman and Wing (1937) have expressed a similar conclusion in their recent publication.

#### SUMMARY

A study has been made of the variants produced by members of the *Escherichia* and *Aerobacter* groups when different species were serially transplanted in sucrose medium. The results may be summarized as follows:

1. The production of sucrose-positive variants is readily accomplished with some strains.
2. An organism which develops very little acid in sucrose medium may give off variants which produce a large amount of acid.
3. There was no difference in the appearance of the original cultures and the variants on eosin-methylene-blue agar.

4. The sucrose-positive variants in a few cases threw off sucrose-negative variants.

5. The sucrose-positive variants retained the power to ferment sucrose after being stored for two years.

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## BACTERIA IN COAL

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Dr. Charles B. Lipman (1931, 1932, 1934, 1935) has reported the occurrence of bacteria in various geological formations, such as ancient rocks, coal, and meteorites, and in old structures erected by man, such as the pre-Inca pyramids in Peru. His explanation of their presence, if correct, has considerable significance. He believes that the bacteria have remained quiescent or dormant in the coal since its formation, i.e., for millions of years and that the bacteria in the meteorites were present before the latter reached the earth's atmosphere.

Coal is believed to have been subjected to various degrees of pressure and heat during its formation. If the bacteria have survived the various stages through which coal passes in its formation, then it follows that either the coal is not subjected to great heat, or, under the conditions of coal formation, bacteria are far more resistant to heat than has been reported under other conditions. Geologists believe, perhaps not unanimously, that some coal formations have been subjected to temperatures as high as 300°C. during formation. This would be for a long period. If bacteria in coal resist such temperatures, then almost the only way to sterilize a piece of coal by heat would be to burn it. It would be a startling phenomenon to find bacteria resisting such high temperatures over indefinitely long periods of time. To meet this objection, Lipman has reported extreme heat resistance of bacteria under experimental conditions. However, in considering Lipman's theory of longevity of bacteria in coal, we must admit that perhaps coal containing bacteria has not necessarily been subjected to great heat. If Lipman's theory of the longevity of bacteria in coal is proven to be correct this

will probably be used as evidence that coal has not been subjected to great heat.

Considering the importance of Lipman's work, it seems strange that no more critical analysis has been given to it. Perhaps this can be accounted for as due to skepticism. The senior author became interested in the problems raised by Lipman in 1932, and has been conducting experiments dealing with the subject somewhat intermittently since that time. The present paper is for the purpose of reporting the results.

Three explanations have been offered to account for isolation of bacteria from geological formations or material of human construction such as ancient building material: (1) the organisms recovered are contaminants due to some failure in technique; (2) they are the result of comparatively recent penetration; (3) they were in the material as formed and remained alive for indefinite periods of time, either dormant or vegetatively active.

Dr. Lipman is a firm believer in the third explanation and favors the view that the organisms have remained dormant. Some formations have contained little moisture, probably not enough for bacterial metabolism. Since bacteria can multiply in surface and distilled water, we may assume that with water present growth might occur.

Lieske (1932) has investigated the occurrence of bacteria in coal and obtained results comparable to that of Lipman.

Farrell and Turner (1932) and Turner (1932) and Farrell (1933) believe the presence of bacteria in coal represents recent penetration and criticize Lipman's work. They found the same organisms in the surface above the mine as in the coal, and after a personal inspection of the mine concluded there was evidence of water penetration. We consider Farrell and Turner are justified in believing that recent penetration as an explanation must be eliminated before we can accept Lipman's hypothesis.

The similarity of organisms in the coal and at the surface is, however, to be expected. Coal is brought to the surface and distributed over the surface of the earth. If organisms can survive in the coal and grow in our test tubes, we can safely assume they can survive on the earth's surface. The similarity or

dissimilarity between organisms in buried geological formations and those on the surface of the earth has no bearing on the question of longevity.

The first explanation, that of poor technique, can safely be eliminated. It is possible to take fifty pieces of brick, heat them red hot to insure sterility, and apply the technique described below, which is comparable to that used by Lipman, and end up with sterile cultures. Contamination can be eliminated.

Our work has been restricted mainly to an investigation of organisms isolated from coal and the penetration of bacteria into various formations. For our work Dr. Lipman very kindly sent us two organisms—a Gram-positive, pink-pigment-producing coccus and a Gram-negative bacillus. Both were non-spore-bearing in cultures.

Since coal is formed under pressure and may be subjected to considerable heat, it seemed advisable to determine the heat resistance of Lipman's organisms. In broth cultures both organisms were killed by pasteurizing temperature. The organisms kept on a dried agar slant for three years were also easily killed by heat. The agar slant was cut in half and the organism recovered from the unheated half, not from the heated. Filterable forms of the Lipman's coccus obtained by growth on Kendall's K medium and filtration were killed by pasteurization. The organism apparently does not produce heat-resistant forms under the conditions of our experiments. They may do so in coal, but that requires an assumption that needs confirmation. Lipman, in support of his stand, believes that all bacteria form resistant cells and has recorded extreme heat resistance in coal.

Some formations containing bacteria apparently contain little moisture over long periods of time. The organisms present, if they survive, must resist desiccation. The Lipman's coccus survived in a dried state in our test tubes and on coal for four years. A number of common non-spore-bearing organisms, such as *Staphylococcus aureus*, *Sarcina lutea*, and *Pseudomonas aeruginosa* survived the same conditions. This is too short a time to have any significance in relation to survival in the coal, but

it indicates they do not die quickly upon drying. The experiment would have had significance only if the organism had died.

Penetration of bacteria into coal, rock, and other formations has a direct bearing on the problem raised by Dr. Lipman. Bacteria pass through filters of diatomaceous earth. There is considerable evidence supporting the belief that they pass through a minute stage in their life cycle. We must consider that as air and water pass through minute pores, bacteria may be carried along.

By connecting a fifteen- to twenty-pound air pressure to glass tubes, cemented in pieces of anthracite and bituminous coal, marine sandstone of miocene age, a recent sedimentary spring deposit, oligocene sandstone, and eocene basalt, and immersing the objects in water, it was possible to demonstrate the passage of air through the objects where no fissures or pores were visible to the unaided eye. Negative results were obtained in some cases, but moving the tube to another point might have produced positive results.

To determine whether bacteria really penetrated coal ten pieces of bituminous and ten pieces of anthracite coal were autoclaved for four hours at fifteen pounds pressure and then placed in cultures of the Lipman coccus and left there for three weeks or longer. The coal was removed, surface sterilization applied, and the coal then placed in flasks of broth. If no growth occurred in three weeks or longer, the pieces were ground up and the powder and small pieces placed in a fresh flask of broth.

The surface sterilization consisted of immersion in 1:500 mercuric chloride or superoxol for ten minutes or longer. This is sufficient for the non-spore-bearing organisms used. Prolonged exposure was avoided. The pieces of coal were immersed for two minutes in two changes of boiling water to remove the bichloride or superoxol. They were then immersed in 95 per cent alcohol and flamed and then placed in broth.

Of the twenty pieces of coal placed in broth after surface sterilization, six produced growth. However, the organism recovered was not the experimental organism but a spore-bearing contaminant. All of these six pieces of coal were treated with

the superoxol which was not as effective as the mercuric chloride for surface sterilization.

The remaining fourteen pieces of coal were ground up and placed in fresh flasks. A pure culture of the experimental organism developed in six of the fourteen flasks. The rest remained sterile.

The results obtained indicate that bacteria may penetrate coal beyond the reach of surface sterilization as applied. Also that they may not grow out to the surface when the coal is placed in broth. The penetration occurred in three weeks. Under natural conditions there is unlimited time to allow for deep penetration.

We do not know that the autoclaving sterilized the coal, but since only the experimental organism was recovered from the interior we believe this to be a justified assumption, although the possibility of its being in the coal and surviving the heating must be considered.

Brick is more porous than coal. Forty pieces were heated red hot for two hours to sterilize, then placed in cultures of four non-spore-bearing organisms—Lipman's coccus, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. After three weeks they were tested in the same manner as the coal. No cultures were recovered. The mercuric chloride and superoxol apparently penetrated the brick as far as the organisms. The sterilizing agents were removed by the washing, not enough reaching the flasks to inhibit growth.

In nature penetration may be aided by the pressure of a column of water. A sterile brick was exposed at one spot to a three- to six-foot column, one-half inch in diameter, of a broth culture of Lipman's coccus. After exposure the brick was sawed into ten pieces and the pieces treated as described. Lipman's coccus was recovered from two of the ten pieces. This was repeated four more times. Only one of the five bricks gave negative results.

The experiment was repeated with a piece of bituminous coal. When cut up Lipman's coccus was recovered from six of ten pieces of coal. It is evident that recent penetration into what

appear to be impervious surfaces may account for the presence of the organisms recovered.

Water passes for a considerable distance through cement walls, and wind through brick and mortar. Many geological formations are more porous. Pieces of coal placed in water for forty-eight hours showed an increase in weight from 0 to 60 per cent. An exchange of air between the inside and outside of porous bodies or bodies with open fractures takes place with changes in temperature such as occur between day and night and winter and summer.

Eighteen pieces of brick were sterilized by heating red hot for two hours and then buried six inches deep in a garden. Six months later they were dug up and treated as in the other experiments. Six of the pieces contained organisms beyond the reach of surface sterilization. Both cocci and bacilli were recovered.

Two bricks were taken from the interior of the base of a large smoke stack that had stood for forty-five years and was being demolished. These bricks were cut up into smaller pieces and a number of the pieces tested for organisms by the method described. A number of different cocci and bacilli were recovered from the interior of the brick.

We do not know that brick as manufactured is sterile. However, a brick maker informed us that ordinary red bricks are heated for days to between 1200° and 2500°F. It appears to us that this should result in the destruction of bacteria in the bricks. It is significant that in our heated brick experiments we recovered only the experimental organism when using mercuric bichloride for surface sterilization.

Dr. Lipman has presented problems that are of great significance, difficult of solution, and that will probably be unsettled for some time. Unfortunately, we cannot examine coal as it is formed. We are satisfied that contamination can be ruled out as a possible explanation of the apparent recovery of bacteria from various geological formations. Recent penetration as an explanation has not been ruled out.

The burden of proof still lies with Dr. Lipman. To dispel reasonable doubt concerning the validity of his hypothesis he

should eliminate penetration as a possible explanation. Incredible penetration is no more difficult of acceptance than incredible longevity and incredible heat resistance and it requires no more support from assumptions. If penetration is eventually ruled out as the explanation, there still remains the problem of whether the organisms have amazing longevity in a quiescent condition or whether they survive only in those environments suitable for carrying on their physiological activities.

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## BACTERIA IN COAL

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Before sending his paper on "Bacteria in Coal" to the editor of this journal Dr. Burke did me the courtesy to send it to me for perusal. He asked that I indicate whether or not his statement of my attitude on the subject is fair, and further that I make suggestions for corrections and references to unpublished literature. I replied to Dr. Burke by suggesting one correction for his discussion, and the inclusion of an important citation of a paper by Lieske. I told him further that since a letter did not lend itself well to a scientific discussion I would defer a reply to his main argument until after his paper had appeared in print. The editor of this journal with Dr. Burke's permission now asks for my reply to Dr. Burke's article and I give it herewith.

It is necessary at the outset to give the reader a view of the background of the present discussion. Unless I have overlooked some papers which may have appeared in the last few years on the problem under discussion only three authors have dealt with this subject in this country, viz., in the order of their publications, the writer of this note, Farrell and Turner, and Burke whose paper occasions this note. In other words, other critics of my work (which formed the basis for the papers by Farrell and Turner and Burke) have no experiential basis for criticism of the work on coal bacteria. When I presented my paper on the bacteria in coal before the general botanical section of the A. A. A. S. at its Cleveland meeting in 1930 Dr. Farrell was present and discussed it briefly before the section and privately with me thereafter. The burden of his criticism was that the organisms which I found must have been contaminants since all his experiments showed coal to be *absolutely sterile*. After my paper appeared in print

Dr. Farrell published a paper to refute my results, but in it he mentioned nothing about finding coal absolutely sterile but stated that he did find bacteria in some specimens of coal and that these organisms had penetrated the coal from the air and the water in the mine from which the coal came, and in which he found similar organisms. He explains the fact that only some specimens of coal contain the organisms on the ground that only the coal which is fissured or cracked contains them and that coal which is free from fissures is sterile. He gives photographs of sections of both kinds of coal which are presumed to prove his point. He does not note, however, that his unfissured coal plainly shows large fissures in the photograph as anyone can see for himself. Therefore, I presume that he means that the pieces which have more fissures permit the penetration of organisms whereas those which have less do not do so. It seems to me that this is a very vulnerable conclusion since even a few fissures should allow penetration of some bacteria if that is the explanation of the observations in question. He considers further that my statement to the effect that there was no water in the vicinity from which my samples come is incorrect. Here we have merely his statement of denial of an assertion made to me by the research chemist of the mine who sent me the slabs of coal with which I worked. Is there any good reason for assuming that Farrell is correct and my informant incorrect? But I shall discuss the question of penetration of bacteria in the coal further below. However that may be, the ghost of contamination is now laid by the observations of all of us. This is done directly and irrefutably by Burke and by me and by implication after a different initial argument by Farrell and Turner.

Let us now examine further into the claims made by Farrell and Turner and by Burke that bacterial penetration into the coal accounts for their presence there. Without making any studies on penetration of bacteria into coal Farrell and Turner merely draw the inference that bacteria in air and water in the mine have penetrated the coal since the same forms are found in both. Why, even if we admit that water has access to the coal in question, which for reasons given above I do not, may it not

be that the water and the air of the mine carry the bacteria which are naturally in the coal rather than the reverse? Burke, however, has actually performed experiments to determine whether or not bacteria penetrate coal and he has a very different basis for his argument from that of Farrell and Turner. By way of answering Burke I would remind the reader that I gave an account in my paper of an experiment in which I exposed for three months thoroughly sterilized coal to a heavy suspension of one of the organisms in active culture which I had isolated from the coal and found no penetration of the organism into the coal. Even Burke states that not all the pieces of coal which he exposed to suspensions of living bacteria did show penetration, but that most of them did. But if that is the case and bacteria in heavy suspensions will not in some instances penetrate coal, how can one expect penetration to be so effective as is claimed by Farrell and Turner and by Burke for enormous thicknesses of coal horizontally and vertically? And yet that is our problem. We have hundreds and even thousands of feet of thickness in the coal measures. Is it reasonable to suppose that air- and water-borne bacteria would penetrate these masses more or less thoroughly? We must remember that any small slab of coal taken anywhere contains some fragments with living bacteria.

As regards Dr. Burke's observations on the factor of high temperatures in our discussion I have two points to make. The first is a point which I have made elsewhere to the effect that a number of geologists whom I have consulted advise me that there is no necessity for assuming the existence of anything more than moderate temperatures in the coal measures. The second is that I have shown high heat resistance of bacteria in their natural state in coal, and that Zettnow has shown much higher heat resistance of certain bacterial spores which he studied. The lack of heat tolerance shown by the organisms in a vegetative state studied by Dr. Burke cannot have any cogency as regards heat resistance by spores. Bacteriological literature is rich in evidence on heat tolerance by spores from a knowledge of which every student of the present subject might profit. If the editor allowed me the space I could present a very interesting discus-

sion thereof. As it is, I must refrain from adding anything further on this subject except to state that if the reader does not believe that bacteria in coal have not been subjected to high temperatures he should master the literature of heat tolerance by bacterial spores.

I proceed now to a discussion of still another set of observations and experiments which I have made and which bear on the question of penetration of bacteria into coal as claimed by Farrell and Turner and by Burke. During the past few years I have been studying old materials of historic or prehistoric known ages in order to see if any bacteria have survived in them. Studies of adobe bricks varying in age from about a century to forty-eight centuries disclose the presence of large numbers of living bacteria in the heart of the bricks. These numbers vary from several thousand to two or three hundred million of living bacteria per gram of dry brick, and that by the use of only one type of nutrient medium. On specialized selective media many other forms of bacteria than those on the peptone soil agar plates have been shown to be present. How did these bacteria penetrate into the adobe bricks in question? Let us take the best type of case for the sake of our discussion out of many which I have studied. In the mission at San Luis Obispo in California there was found some time ago a room which had been forgotten for a century. The room had been sealed and was at one time a prison for soldiers. In this room there are found several very wide adobe brick walls from three to five or six feet in thickness and high enough in some cases to reach up to the clay tile roof above the mission. There is not the slightest sign of dripping water from the roof. The mission was built about 160 years ago. By taking bricks from the heart of the wall considered vertically and horizontally, and others from the outer part of the wall as controls, placing them for two or three minutes in hot paraffine at 195°C. and then removing the heart of the brick for study the following results were obtained. Hundreds of millions of living bacteria per gram of the heart of the brick (peptone soil extract agar) were found in both the inner bricks and the outer bricks. Blue-green algae were found only in the outer bricks. Were

hundreds of millions of bacteria per gram of brick driven into the brick in the inside of the wall by the air currents in a perfectly dry and protected room where water could not have played any part? If so, why weren't the blue-green algae (*Nostoc* and others) blown into the brick in the heart of the wall along with those numerous bacteria? Would air currents carry bacteria in uniform numbers to all parts of every brick? And yet we know that all the bricks studied give the same results. It is, of course, true that bricks 160 years old are not in the same case as those which are 5000 or 10,000 years old, but my results show numerous bacteria (perhaps in thousands per gram) in bricks which are forty-eight centuries old. In other words, some bacteria are much more resistant to desiccation as well as heat and for much longer periods than others and they would be the forms found in the oldest historic or geologic material. That this is a plausible assumption is attested by the studies of K. F. Meyer and his associates on heat resistance of *Clostridium botulinum* spores and those of the flat-sour types. Unfortunately space in this paper will not permit my presenting additional cogent evidence against the theory of penetration of bacteria into rocks and coal as advocated by Burke and others. It is gratifying that all others agree with me that bacteria are found in coal even though they do not interpret their findings as I do mine. In this connection I cannot refrain from directing attention to the fact that no one of my American critics has mentioned the work of Lieske on bacteria in coal even though his studies have been much more extensive than any other investigator's both in point of time and in variety of material. I was obliged to call the attention of even my friend Dr. Burke to Lieske's investigations and especially to the article in which nearly five years ago Lieske gave confirmation to my results, obtained quite independently and differing with me only as regards how the bacteria in the coal maintain themselves there in a living state. Since Lieske is one of Germany's most versatile and distinguished microbiologists it is surprising that my American critics are unaware of his work.

When I have prepared for publication the vast amount of ex-

perimental data which I have gathered in the last decade I shall be able to convince everyone of the correctness of the points of view expressed above. Meanwhile I proceed with my investigations never more buoyant and encouraged in my position in the controversy than now.

In closing this brief reply to Dr. Burke and his school of thought which I presume is all which I can be allowed now, I wish to express my sincere appreciation of Dr. Burke's courtesy and gentlemanly attitude in our correspondence. He has evinced the attitude of the true scientist and has my high respect.

# THE RELATION OF CERTAIN RESPIRATORY ENZYMES TO THE MAXIMUM GROWTH TEMPERATURES OF BACTERIA

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Certain bacteria are characterized by ability to perform vital functions necessary for their reproduction at temperatures which are decidedly inhibitory to most organisms. Some of these resistant forms grow normally at temperatures well above 60°C. Although some strains or types of non-sporulating organisms, as, for example, certain sarcinae, staphylococci, streptococci and lactobacilli, have been referred to as thermophilic, the most common and generally recognized thermophiles are spore-forming rods which resemble to a considerable extent certain members of the genus *Bacillus*.

Miquel (1879-1888) was perhaps the first to isolate a strictly thermophilic bacterium. He believed that this organism, which grew well at 70°C., contained a form of protoplasm different from that found in ordinary bacteria. From this time on there has been much speculation regarding the principles governing thermophilism.

Dallinger (1887) reported that a certain flagellate which normally could withstand a temperature of only 18°C. adapted itself over a period of seven years to normal growth at a temperature of 70°C. During this process of adaptation a change in vacuolization was noted, with a concurrent loss of moisture content. Davenport and Castle (1896) found that tadpoles hatched from eggs incubated at temperatures from nine to ten degrees higher than normal could withstand a temperature three

<sup>1</sup> This paper covers in part a dissertation submitted to the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.



degrees above that ordinarily tolerated. Magoon (1926) and Williams (1936) developed a slightly more heat resistant strain of *Bacillus mycoides*, by selection.

Water may play a considerable rôle in the ability of thermophilic organisms to resist heat. Benecke (1912) claimed to have demonstrated that bacterial spores contain less moisture than do vegetative cells. This suggested the possibility of desiccation accounting for increased thermal resistance of spores. Fluegge (1886) and others found that spores are more easily killed by heat in water than in air. The common knowledge that moist heat is more lethal than dry heat further suggests the possible rôle of water. However, Virtanen and Pulkki (1933) held that spores of *Bacillus mycoides* do not possess a lower moisture content than do vegetative cells of this species.

von Esmarch (1888) believed that bacterial spores have a firm protective coating which partially prevents passage of heat. Lewith (1890) did not regard this assumption as plausible, because thickness of the coating would be insignificant in its protective effect. Virtanen (1934) computed that the insulating property of the spore wall would have to be a million times greater than that of ordinary air, in order to exert a definite protective effect. Lewith demonstrated that the coagulation temperature of pure albumin (egg) is inversely proportional to moisture content. Robertson (1927a) demonstrated an increased thermal resistance for various organisms in hypertonic solutions. This author (1927b) concluded that ability of living protoplasm to survive high temperatures may depend in part upon a low cell-moisture content, and presented data which suggested that (1) changes in the nature of the cell-wall membrane, (2) changes in the cells produced by growth in concentrated sucrose solutions, and (3) changes evolved through acclimatization processes, may be instrumental in producing cells which have a low moisture content and a consequent increase in thermal resistance.

Rabinowitsch (1895) observed that growth of certain thermophilic bacteria on agar and broth was more luxuriant under anaerobic than under aerobic conditions at lower temperatures, while at higher temperatures the reverse was true. Schuetze

(1908) and Ambroz (1910-11) confirmed her findings, which indicated the possibility of oxidation-reduction playing a rôle in thermophilic growth.

Virtanen suggested that an increased thermal resistance of spores over that of vegetative cells may be attributed to a firmer combination of enzyme and protein within the spore.

Casman and Rettger (1933) were unsuccessful in attempts to acclimate certain spore-forming bacilli to temperatures appreciably higher than the original maximum. Neither did desiccation or growth in concentrated solutions of sucrose, peptone or sodium chloride result in increased heat tolerance. However, with nine common members of the genus *Bacillus* and three strictly thermophilic organisms they observed what appears to be a distinct correlation between maximum growth temperatures of the organisms and minimum temperatures of destruction of certain of the bacterial respiratory enzymes.

The promising results of the last-named investigators stimulated the present writers to conduct a similar, but more extensive, study of the relationship of certain respiratory enzymes to the growth of bacteria at relatively high temperatures.

Most, if not all, of the respiratory processes of bacteria are assumed to be accomplished through the intervention of enzymes. Waldschmidt-Leitz (1929) defined enzymes as specific organic catalysts formed by living cells, but independent of the vital integrity of the cells. Stephenson (1930) stated that bacterial respiration covers any chemical process in which energy is liberated which can be utilized by the cell. Reactions can take place either by activation of molecular oxygen or of any oxidizable substance.

In this paper the term "enzyme" as applied to respiratory mechanisms of bacteria is employed for convenience, and is not used in the older and more restricted sense.<sup>2</sup>

Each of the common bacterial respiratory systems<sup>3</sup> contains

<sup>2</sup> See Ph.D. dissertation of the senior author in the Yale University Library for description of methods, and for more extensive discussions than are presented here.

<sup>3</sup> Stephenson (1930) reviews the various theories of bacterial respiratory systems.

at least one thermolabile enzyme, destruction of which would presumably inhibit proper functioning of the system. It seems reasonable to assume that, in order to grow, bacteria must respire. Any interference with the mechanism of respiration would necessitate either substitution of a different respiratory system or loss of the ability to respire. The last-mentioned possibility should result in cessation of growth processes.

#### EXPERIMENTAL

In the present investigation 104 organisms were employed, including 11 that were used by Casman and Rettger (1933), and various strains from the stock culture collection of this laboratory and from those of several other institutions; also some isolations from hay, potatoes, soil, water, and leaves of various flowering plants and trees.

Throughout the experimental work a medium of the following composition was employed, unless otherwise noted.

Proteose-peptone, Difco.....	5 grams
Bacto-beef extract, Difco.....	3 grams
Distilled water.....	1000 cc.

To obtain a solid medium, 15 grams of Difco granulated agar were added to this basal medium. Various carbohydrates used were added in final concentrations of 1.0 per cent.

Preliminary observations showed that some strains were improperly labelled. While it was not necessary for an evaluation of experimental results that the taxonomic position of each strain be known, it was essential to separate cultures into different groups according to their physiological, morphological, and cultural characteristics, in order to determine possible group relationships between their maximum growth temperatures and minimum temperatures of destruction of certain respiratory enzymes. It was postulated that such a relationship, if it could be established, should help to explain thermophilic growth.

All strains were subjected to an extensive routine examination, to determine their purity and, whenever possible, their exact taxonomic position. No appreciable tendency to variation was noted in any established strains during the investigation.

By morphological, cultural, and physiological tests the final collection of stock cultures was divided into 17 groups, in addition to a group of real thermophiles. The number of organisms in each group varied from 1 to 21. In a large majority of instances strains within a group were quite similar, within narrow limits.

Twelve groups could be identified as known species of the genus *Bacillus*. The remaining groups, all Gram-positive, aerobic, spore-forming rods, could not be placed in any particular species of this genus, although their characteristics were sufficiently different to warrant separation into groups.

The Marburg strain of *Bacillus subtilis* was selected as the typical *B. subtilis* strain, whereas the Michigan strain (Soule, 1932) was placed with the *Bacillus cereus* type, which it more closely resembles, according to Conn's (1930) and our observations. The Marburg type of *B. subtilis* and other strains which closely resembled it were consistently Voges-Proskauer-positive and methyl-red-negative, while the reverse was true of representatives of the *B. cereus* type.

#### DETERMINATION OF MAXIMUM GROWTH TEMPERATURES

Maximum temperatures permitting macroscopic growth within 24 hours were determined in broth, agar slants and in glucose-agar shake tubes, all adjusted to pH 7.2. This information was regarded as important for several reasons: (1) to determine whether or not the type of culture medium is an important factor in studies of this kind, (2) to determine significant differences in weighted mean maximum growth temperatures of various groups of organisms, and (3) to measure the degree of correlation, if any, between these temperatures and minimum temperatures of destruction of certain respiratory enzymes.

Multiplicate tubes of each medium previously heated to the temperature of incubation were seeded with 24-hour subcultures (grown at optimum temperature) of each strain. They were immediately placed in incubators in racks allowing ample circulation of air around the culture tubes. After 24 hours' incubation at given test temperatures they were examined for macro-

scopic evidence of growth, that is, clouding in broth, visible growth on agar slants, and colonies in shake tubes.

These criteria of growth were arbitrarily chosen because of their common use in many routine bacteriological tests. They, in themselves, ignore, of course, the possibility of cell growth and increase in cell size without increase in number, or in sufficient volume to be visible to the naked eye. They also exclude multiplication at extremely low rates at higher temperatures, similar to what Hansen (1933) noted in the growth of thermophiles at and below room temperature.

Variations in results on different occasions and within simultaneously treated replicate sets indicated desirability of statistical methods as a means of deriving the most probable value for the maximum growth temperature of each strain. Some factors that may have been responsible for variations were not studied. Undoubtedly they included differences in incubators, media, individual strains, and errors of observation.

Determinations of maximum growth temperatures of all stock strains required approximately 6,000 test tubes of media. The weighted mean maximum growth temperature was computed from the results for each strain grown on each of the three media. The hypothesis upon which computation of these values depends is stated here.

If a tube of medium inoculated with the test organism is incubated considerably below the maximum growth temperature, growth may reasonably be expected to occur, no matter how many times the procedure is repeated. Conversely, at temperatures well above the critical point growth would never occur in spite of repeated trials.

However, as the temperature of incubation approaches the region of maximum growth temperature from either of the two directions, the factors causing variation possess relatively greater magnitude, become operative and cause the chances of a positive or negative result to become equal, or as one to one. Thus, the concept of maximum growth temperature is not that of a hair-line division immeasurably small, but rather that of a range from one to several degrees in extent. The weighted mean of

this range is the most probable value for the critical point desired, that is, the maximum growth temperature.

Accordingly, the range may be defined as the difference in degrees Centigrade between the lowest and highest temperatures for which both positive and negative tests are found within a series of multiplicate trials. Either positive or negative test

TABLE 1

*Weighted mean maximum growth temperatures (with standard deviations) of the groups of stock strains*

PROBABLE CLASSIFICATION	NUMBER OF STRAINS	WEIGHTED MEAN MAXIMUM GROWTH TEMPERATURE (IN SLANT AGAR, AGAR SHAKE & BROTH TUBES)
		°C
<i>Bacillus mycoides</i> . . . . .	4	40.0 $\pm$ 3.1
<i>B. prausnitzii</i> . . . . .	1	40.0
Unidentified . . . . .	5	41.8 $\pm$ 1.5
<i>B. simplex</i> . . . . .	3	43.3 $\pm$ 0.9
<i>B. cereus</i> . . . . .	21	44.7 $\pm$ 1.9
<i>B. megatherium</i> . . . . .	8	46.0 $\pm$ 1.0
<i>B. tumescens</i> . . . . .	1	46.0
<i>B. alvei</i> . . . . .	3	46.3 $\pm$ 1.5
<i>B. petasites</i> . . . . .	3	46.3 $\pm$ 0.5
Unidentified . . . . .	4	48.3 $\pm$ 0.8
<i>B. niger</i> . . . . .	1	49.0
Unidentified . . . . .	3	51.3 $\pm$ 0.5
Unidentified . . . . .	5	52.4 $\pm$ 0.5
<i>B. subtilis</i> . . . . .	10	54.3 $\pm$ 4.6
<i>B. vulgatus</i> . . . . .	5	55.0 $\pm$ 2.5
Unidentified . . . . .	6	56.8 $\pm$ 2.2
<i>B. mesentericus-fuscus</i> . . . . .	12	57.7 $\pm$ 2.2
Thermophiles . . . . .	9	75.6 $\pm$ 1.1
Total . . . . .	104	

figures may be used in computation, as both give similar results; arbitrarily, the former have been chosen.

The weighted mean in this instance is the arithmetical average of this range. In computing this value the relative effect of each item on the final result is considered. Weighted mean maximum growth temperatures were computed for each strain for each medium.

To discover the effect of varying culture medium upon upper limits of growth, the weighted mean maximum growth temperature was computed for the entire collection of strains for each medium (broth, agar slant and agar shake tubes). No significant difference could be demonstrated, whether determinations were made in broth, on agar slants, or in agar shake tubes. The value for the weighted mean maximum growth temperature of all test organisms in each of the three media was 51.5°C.

Thus, physical state of medium (liquid or solid) apparently did not affect the upper temperature limit of growth. Exposure to atmospheric oxygen on agar slant cultures did not raise or lower the value for the upper limit of growth as determined in broth or shake cultures.

However, when strains were classified, as shown in table 1, into 18 groups (including one group of thermophiles) according to their morphological, physiological and cultural characteristics, and the weighted mean maximum growth temperature was computed for each group, significant differences were found among certain groups, but not among values found by use of the three media for any one group.

Within the single genus, *Bacillus*, wide differences were found in upper limits of temperature-tolerating growth. These differences were especially marked between the first five and the last five groups (excluding the thermophiles).

#### MINIMUM TEMPERATURES OF DESTRUCTION OF RESPIRATORY ENZYMES

From numerous thermolabile enzymes which have been referred to as performing rôles of more or less importance in respiratory mechanisms of bacteria, indo-phenol oxidase, catalase and succino-dehydrogenase were selected for further study, because earlier work in the laboratory had indicated a definite relationship between their minimum temperatures of destruction and maximum temperatures of growth of certain bacteria.

Although tests for these three enzymes, and for peroxidase and hydrogen peroxide, were performed as nearly simultaneously on each culture as experimental procedure permitted, they will be

discussed separately. A uniform procedure for all experimental work was devised, in order that results with different types of strains would be comparable.

Determination of thermolabilities of various enzymes are most frequently made at relatively short periods of exposure, seldom of more than one-half hour's duration. If longer periods had been used in this study, the minimum temperatures of destruction for enzymes would not be comparable to those found by earlier workers. As 24 hours' exposure was selected for maximum growth temperature studies, and as growth is dependent, in part at least, upon proper functioning of respiratory enzymes, the same period was adopted for determinations of minimum temperatures of destruction of these enzymes. Certain data obtained, but not included here, showed the wisdom of this choice.<sup>4</sup>

Optimum pH for various tests was established by experimentation. In general, reagents and enzymes were not particularly sensitive to changes in pH, and rather wide ranges were found to be satisfactory for tests.

Influence of different concentrations of bacterial cells upon the tests was also studied. Suspensions of test organisms were prepared having turbidities varying from no. 0.5 to no. 6.0, McFarland's barium sulphate nephelometer standard. Practically identical results were obtained at various concentrations. A turbidity of no. 2.0 was adopted.

The method of Quastel and Whetham (1924) for preparing so-called "resting bacteria" was used for obtaining a suspension of test organisms. This was considered advisable for this study, because the reactions are uncomplicated by analytic or synthetic growth processes.

Tubes of nutrient broth were inoculated with the stock strains to be tested. After 24 hours' incubation the cultures were poured aseptically over the surface of nutrient agar in Kolle flasks. At the expiration of 24 hours' incubation at the optimum temperature the organisms were removed and suspended in sterile distilled water. The suspensions were centrifuged and washed

<sup>4</sup> Ph.D. dissertation of the senior author in the Yale University Library.



twice (preliminary studies showed further washing and aeration to be unnecessary). The cell suspensions were then diluted to the desired turbidity. After dividing each suspension into eight aliquot portions, the different portions were subjected to different temperatures for 24 hours. The temperature range was 35°C. to 70°C., with five degree intervals. The following tests were then carried out with each of the eight tubes.

#### ENZYME TESTS

##### *Indo-phenol oxidase*

Keilin (1928-29) showed that oxidase activity in yeasts could be measured by using para-phenylenediamine-hydrochloride as a test reagent. Casman and Rettger (1933) employed the following method for detecting the presence of bacillary indo-phenol oxidase:

Five tubes were required for each strain for each selected temperature. To each tube was added 0.5 cc. potassium dihydrogen phosphate-sodium hydroxide buffer, pH 7.3 (Clark, 1922) and 0.5 cc. bacterial suspension. Tube 1 was not heated; tube 2 was boiled for 15 minutes; tube 3 received potassium cyanide in a final concentration of 0.01 per cent; to tube 4 was added 0.1 cc. of 3.0 per cent hydrogen peroxide; and to tube 5 three drops of a freshly prepared water extract of horseradish (peroxidase). When tube 2 was cool, 0.5 cc. of a 1.0 per cent solution of para-phenylenediamine-hydrochloride, buffered at pH 7.3 and prepared immediately before use, was added to each tube. Following thorough mixing, the tubes were allowed to remain undisturbed; at definite intervals the degree of coloration was noted. Production of an intense brownish-purple color in tube 1 was positive evidence of the presence of indo-phenol oxidase, providing tubes 2 and 3 were negative. Tubes 4 and 5 served as tests for peroxidase and hydrogen peroxide, respectively. The last two tubes, if positive, were significant only when tube 1 was negative.

##### *Peroxidase*

Callow (1926) found that bacterial peroxidase is thermostable. Indeed, boiling the bacterial suspensions and cooling before add-

ing the test reagents increases both rapidity of appearance and intensity of color produced with this reagent.

Farrell (1935) found that tincture of o-tolidine and an aqueous solution of 2,7 diamino-fluorene-hydrochloride were very sensitive reagents for determining the presence of peroxidase of streptococci. Callow (1926) used guaiac and benzidine for detecting this enzyme.

Schmidt and Hinderer (1932) suggested the use of 2,7 diamino-fluorene-hydrochloride as a valuable reagent for detection of blood, pasteurized milk, etc., because of its increased solubility and activity as compared with that of benzidine. Indeed, one drop of blood in 50,000 cc. of water gave a strong positive test with 2,7 diamino-fluorene-hydrochloride, while benzidine failed to react. Since this new compound could not be obtained from any known source, it was prepared in this laboratory by the method of Schmidt and Hinderer (1931).

The four above-mentioned test reagents were used as follows in this investigation for detecting peroxidase: (1) 1.0 per cent guaiac in 95 per cent alcohol, (2) 0.5 per cent benzidine in 50 per cent alcohol, (3) 1.0 per cent o-tolidine in 50 per cent alcohol, and (4) 0.1 per cent 2,7 diamino-fluorene-hydrochloride in water to which a trace of levulose was added.

One-tenth cubic centimeter of color reagent was added to a mixture of 0.5 cc. cell suspension, 0.5 cc. potassium hydrogen phthalate-sodium hydroxide buffer (pH 4.5) and 0.1 cc. of 3.0 per cent hydrogen peroxide. Both heated (boiled for 15 minutes) and unheated suspensions were tested. Appearance of a characteristic blue color constituted a positive test.

Substitution of three drops of fresh water extract of horseradish (peroxidase) in place of hydrogen peroxide served as a test reagent for peroxide.

#### CATALASE

Catalase differs from peroxidase in its action on hydrogen peroxide by producing a direct evolution of oxygen. Its presence was detected by adding 0.1 cc. of 3.0 per cent hydrogen peroxide to a mixture of 0.5 cc. cell suspension and 0.5 cc. potas-

sium dihydrogen phosphate-sodium hydroxide buffer (pH 7.3). Evolution of gas bubbles constituted a positive test for catalase.

### *Succino-dehydrogenase*

Methylene blue was used as indicator for succino-dehydrogenase activity of cells under anaerobic conditions. Two-tenths cubic centimeter of a 1:40,000 sterile solution of the dye was added to a mixture of 0.5 cc. cell suspension, 0.5 cc. sterile potassium dihydrogen phosphate-sodium hydroxide buffer (pH 7.3) and 0.3 cc. sterile 5.0 per cent neutralized succinic acid. The tubes were fastened by means of a rubber band around a beaker filled with cotton to give a white background. They were placed in a Novy Jar which was evacuated and incubated at 37°C. Readings were made of the degree of decolorization at convenient intervals.

### RESULTS

A thermostable peroxidase was detected in all strains with each of the four color reagents. In no instance was hydrogen peroxide detected, although strong positive tests were given by  $m/10,000$  dilutions of hydrogen peroxide as a control. This was expected, because of the presence of a strong catalase activity in each strain.

Thermolabile indo-phenol oxidase, catalase and succino-dehydrogenase were found in every bacterial strain; however, the degree of heat resistance of these respiratory enzymes varied with the different bacterial strains. A typical set of data is given in table 2. Fairly close agreement between maximum growth temperature (58°C.) and minimum temperature of destruction of three enzymes, indo-phenol oxidase, catalase and succino-dehydrogenase (all 60°C.) is shown in this table.

In order to generalize upon this specific example by employing the data for all stock strains, weighted mean minimum temperatures of enzyme destruction were computed for each of the 17 groups of aerobic spore-formers, and for the thermophiles. It will be seen in table 3 that some degree of relationship exists between the two variables.

Statistical treatment of data made possible a determination of the degree of relationship, and permitted its expression in simple mathematical terms.

TABLE 2

*A typical example of determinations of minimum temperatures of destruction of indo-phenol oxidase, catalase, and succino-dehydrogenase*

	CONTROL		TUBE NUMBER					
			1	2	3	4	5	6
Temperature of exposure..			45°C.	50°C.	55°C.	60°C.	65°C.	70°C.

## Indo-phenol Oxidase

TIME OF READING			TUBE NUMBER									
			+	-	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
minutes												
15	-	-			----	----	----	----	----	----	----	----
45	2	-			2-- 4 2	2-- 4 2	2-- 4 2	2-- 4 2	2-- 4 2	2-- 4 2	2-- 4 2	2-- 4 2
150	4	+			4++ 4 3	4++ 4 3	4++ 4 3	4++ 4 3	4++ 4 3	4++ 4 3	4++ 4 3	4++ 4 3

1 = indo-phenol oxidase, 2 = heated control, 3 = KCN control, 4 = peroxidase, and 5 = hydrogen peroxide.

## Catalase

TIME OF READING	+	-	TUBE NUMBER					
			1	2	3	4	5	6
minutes								
15	+	-	+	+	+	-	-	-
60	+	-	+	+	+	-	-	-

## Succino-dehydrogenase

TIME OF READING	+	-	TUBE NUMBER					
			1	2	3	4	5	6
<i>minutes</i>								
15	4	—	—	—	—	—	—	—
30	4	—	4	4	4	—	—	—
60	4	—	4	4	4	—	—	—

- negative, + positive, and 1, 2, 3, 4, various degrees of positiveness.

Maximum growth temperature = 55°C.

Mean maximum growth temperatures as *X* values (independent variable) for each of the 18 groups, and mean minimum temperatures of enzyme destruction as *Y* values (dependent variable)

for indo-phenol oxidase, catalase, and succino-dehydrogenase were plotted (figs. 1, 2, and 3).

Arrangement of points suggested a straight-line relationship between the two variables for each enzyme. Consequently, equations for lines of regression of  $Y$  on  $X$  (solid lines on graphs)

TABLE 3

*The relation of the maximum growth temperatures of the stock strains to the minimum temperatures of destruction of certain enzymes for the eighteen groups*

PROBABLE CLASSIFICATION	NUMBER OF STRAINS	MAXIMUM GROWTH TEMPERATURE	MINIMUM TEMPERATURE OF DESTRUCTION		
			Indo-phenol oxidase	Catalase	Succino-dehydrogenase
		°C	°C	°C	°C
<i>Bacillus mycoides</i> .....	4	40	41	41	40
<i>B. prausnitzii</i> .....	1	40	44	40	40
Unidentified .....	5	42	52	54	49
<i>B. simplex</i> .....	3	43	55	52	40
<i>B. cereus</i> .....	21	45	48	46	50
<i>B. megatherium</i> .....	8	46	48	50	47
<i>B. tumescens</i> .....	1	46	55	46	46
<i>B. alvei</i> .....	3	46	51	50	53
<i>B. petasites</i> .....	3	46	47	51	45
Unidentified .....	4	48	50	54	57
<i>B. niger</i> .....	1	49	65	65	50
Unidentified .....	3	51	58	55	52
Unidentified .....	5	52	57	57	53
<i>B. subtilis</i> .....	10	54	60	56	51
<i>B. vulgatus</i> .....	5	55	56	56	50
Unidentified .....	6	57	56	64	55
<i>B. mesentericus-fuscus</i> .....	12	58	60	63	53
Thermophiles .....	9	76	65	67	59

for the three enzymes, indo-phenol oxidase (1), catalase (2) and succino-dehydrogenase (3), namely

$$Y = 23.0 + 0.59X \quad (1)$$

$$Y = 19.9 + 0.66X \quad (2)$$

$$Y = 31.8 + 0.36X \quad (3)$$

were fitted by the method of least squares. Lines constructed from these equations fit the data more closely than any other line that could be drawn through the array of points.

These lines picture the average relationship between the two variables for each enzyme. They show graphically the average change in minimum temperatures of destruction, (dependent variable) which accompanies or is produced by a given unit change in maximum growth temperatures (independent variable). Equations of these lines are called equations of regression.

Indo-phenol Oxidase  
in °C.

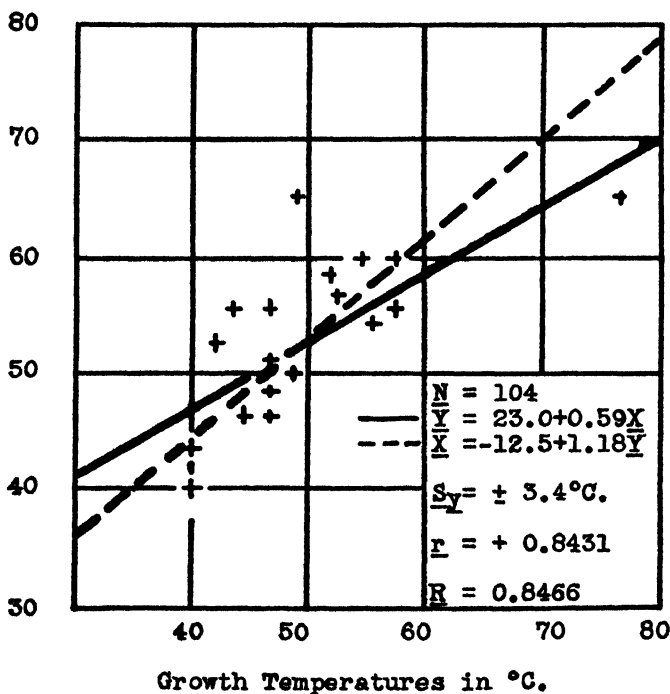


FIG. 1. THE RELATION OF THE MAXIMUM GROWTH TEMPERATURES OF THE STOCK STRAINS TO THE MINIMUM TEMPERATURES OF DESTRUCTION OF INDO-PHENOL OXIDASE

$S_y$ , the standard error of estimate, is a measure of the closeness of the plotted points to the line of regression. If a value for this factor is large, points in general do not lie close to the line and the degree of relationship is not high. Conversely, if  $S_y$  has a low numerical value, points in general lie close to the line, and the degree of relationship is high. In the latter instance reli-

ability of estimates made with the equation of regression is the greater. Sixty-eight per cent of all observations will lie within the range  $\pm S_y$  in a normal distribution.

For indo-phenol oxidase a value of  $\pm 3.35^\circ\text{C}.$ , for catalase  $\pm 4.0^\circ\text{C}.$ , and for succino-dehydrogenase  $\pm 3.0^\circ\text{C}.$  was obtained.

Catalase  
in  $^\circ\text{C}.$

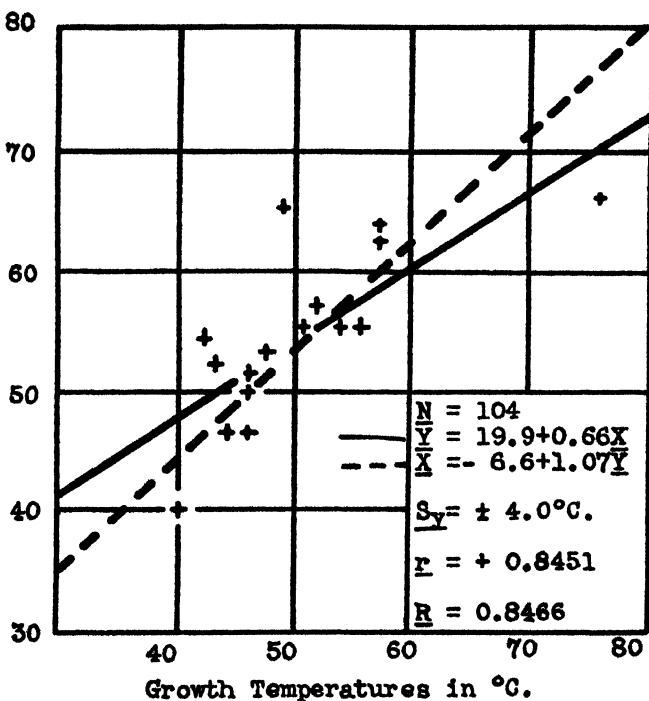


FIG. 2. THE RELATION OF THE MAXIMUM GROWTH TEMPERATURES OF THE STOCK STRAINS TO THE MINIMUM TEMPERATURES OF DESTRUCTION OF CATALASE

These values expressed the degree of dispersion of points about the line of regression. Furthermore, in 68 out of 100 instances minimum temperatures of destruction of indo-phenol oxidase as estimated by equation (1), of catalase (2), and of succino-dehydrogenase (3) will not differ from observed values by more than  $\pm 3.35^\circ\text{C}.$ ,  $\pm 4.0^\circ\text{C}.$ , or  $\pm 3.0^\circ\text{C}.$ , respectively.

It will be noted that this factor,  $S_y$ , is expressed in original

units (degrees Centigrade) of measurement of the  $Y$  variable. In many instances it would be more desirable to use an abstract measure which, not being expressed in original units of measurement, would be applicable for comparing data in units of different kind or magnitude.

Succino-dehydrogenase  
in °C.

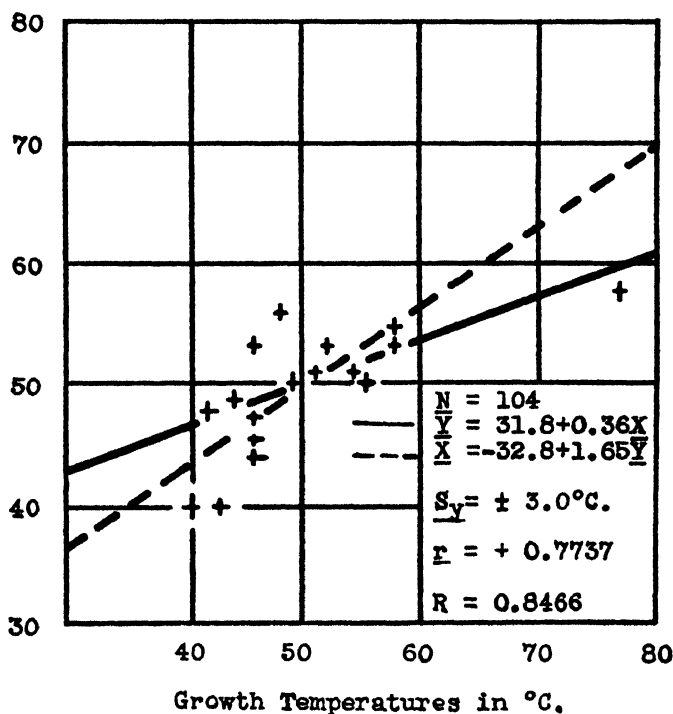


FIG. 3. THE RELATION OF THE MAXIMUM GROWTH TEMPERATURES OF THE STOCK STRAINS TO THE MINIMUM TEMPERATURES OF DESTRUCTION OF SUCCINO-DEHYDROGENASE

The coefficient of correlation,  $r$ , is such a device. This factor expresses abstractly the relative degree of relationship between two variables, regardless of size or kind of units of measurement used in mensuration or computation of either variable.

For perfect correlation,  $r$  has a value of 1.0. Where no correlation exists a value of 0.0 is obtained. For direct correlation



the factor is generally written  $+1.0$ , for inverse correlation  $-1.0$ .

Values obtained for coefficients of correlation,  $r$ , were: indo-phenol oxidase  $+0.8431$ , catalase  $+0.8451$ , and succino-dehydrogenase  $+0.7737$ . These express, in a single figure, the full degree of relationship (which must be considered as high) between the minimum temperatures of destruction of the three enzymes individually and the maximum growth temperatures of bacteria.

The advantages of a simple expression which states the degree of such relationship as accurately, and much more simply, than does any table with a large number of recorded observations is apparent. General trend is shown, although at a sacrifice of non-essential detail.<sup>5</sup>

It should be pointed out that for existence of a high degree of relationship it is not necessary that each pair of maximum growth and enzyme minimum destruction temperatures be identical, but only that increments in maximum growth temperatures be accompanied by proportional increases in minimum temperatures of destruction of enzymes.

A possible objection to paucity of strains in certain groups was set aside by giving proper weight to each term in the statistical treatment.

To measure the effect of the three factors collectively upon maximum growth temperatures of bacteria, use was made of the coefficient of multiple correlation,  $R_{1.234}$ . This factor expresses degree of relationship between three independent variables (minimum temperatures of destruction of indo-phenol oxidase (2), catalase (3), and succino-dehydrogenase (4)) collectively, and the maximum temperatures of growth of organisms (1).

In computing this index the effect that each factor exerts on maximum growth temperature is combined by the method of calculation with that of the other two. In this instance the value for  $R_{1.234}$ ,  $0.8466$ , indicated that a high degree of relationship exists, and, disregarding possible concealed factors, that

<sup>5</sup> For a discussion of theory and derivations of the above statistical measures, Mills (1924) or any other standard work on statistics may be consulted.

enzyme temperatures of destruction may possibly play an important rôle in controlling maximum growth temperatures of bacteria.

#### THE PROBLEM OF ESTIMATION

Another application of equations of regression fitted by the method of least squares is to the problem of estimation. Substitution of observed values for maximum growth temperatures of single strains of organisms, or of groups of strains in proper equations of regression of  $Y$  on  $X$ , permitted computation of the most probable value for an unknown minimum temperature of destruction of an enzyme.

Conversely, maximum temperatures of growth can be computed if minimum temperatures of destruction of enzymes are known, by employing the equation of regression of  $X$  on  $Y$ .

By the method of least squares, equations

$$Y = 23.0 + 0.59X \quad (1)$$

$$Y = 19.9 + 0.66X \quad (2)$$

$$Y = 31.8 + 0.36X \quad (3)$$

where  $Y$  is the minimum temperature of destruction of the enzyme, and  $X$  is the maximum growth temperature of the organisms, as the independent variable, were fitted to the data. These regression equations respectively express the average relationship between the two factors for the three enzymes, indo-phenol oxidase (1), catalase (2) and succino-dehydrogenase (3).

For 21 strains of *B. cereus* weighted mean maximum temperatures of growth and weighted mean minimum temperatures of destruction of indo-phenol oxidase had values of  $45 \pm 1.9^\circ\text{C.}$  and  $48^\circ\text{C.}$ , respectively.

If the first-named value is substituted for  $X$  in equation (1), upon solution the value

$$Y = 50.0^\circ\text{C.}$$

is obtained, which is two degrees more than the observed value.

The differences recorded in table 4 were computed in a similar manner. In only a few instances were the differences greater than five degrees, and in nine, within 3 degrees. This approach to close agreement between observed and computed values was a further check on the reliability of equations of regression of  $Y$  on  $X$ . Magnitude of differences between the two values,

TABLE 4

*Relations between observed and computed values of the minimum temperatures of destruction of the respiratory enzymes*

PROBABLE CLASSIFICATION	MAXIMUM GROWTH TEMPERATURE	INDO-PHENOL OXIDASE $Y = 22.0 + 0.59X$			CATALASE $Y = 19.9 + 0.66X$			SUCCINO-DEHYDROGENASE $Y = 31.8 + 0.36X$		
		Observed	Computed	Difference	Observed	Computed	Difference	Observed	Computed	Difference
	°C									
<i>Bacillus mycoides</i> .....	40	41	47	+6	41	46	+5	40	46	+6
<i>B. prausnitzii</i> .....	40	44	47	+3	40	46	+6	40	46	+6
Unidentified.....	42	52	48	-4	54	48	-6	49	48	-1
<i>B. simplex</i> .....	43	55	49	-6	52	48	-4	40	47	+7
<i>B. cereus</i> .....	45	48	50	+2	46	49	+3	50	48	-2
<i>B. megatherium</i> .....	46	48	50	+2	50	50	0	47	48	+1
<i>B. tumescens</i> .....	46	55	50	-5	46	50	+4	46	48	+2
<i>B. alvei</i> .....	46	51	50	-1	50	50	0	53	48	-5
<i>B. pasteurii</i> .....	46	47	50	+3	51	50	-1	45	48	+3
Unidentified.....	48	50	51	+1	54	52	-2	57	49	-8
<i>B. niger</i> .....	49	65	52	-13	65	52	-13	50	49	-1
Unidentified.....	51	58	53	-5	55	53	-2	52	50	-2
Unidentified.....	52	57	54	-3	57	54	-3	53	50	-3
<i>B. subtilis</i> .....	54	60	55	-5	56	55	-1	51	51	0
<i>B. vulgaris</i> .....	55	56	56	0	56	56	0	50	51	+1
Unidentified.....	57	56	57	+1	64	57	-7	55	52	-3
<i>B. mesentericus-fuscus</i> .....	58	60	57	-3	63	58	-5	53	52	-1
Thermophiles.....	76	65	70	+5	67	67	0	59	59	0

observed and computed, compared very favorably with the 5° unit interval of measurement of enzyme destruction temperature determinations.

Within limits of the graph, 40° to 75°C., values of enzyme destruction temperature could be estimated nearly as accurately as they could be measured. Beyond this range, where controlling

factors may be different, such computations could not be made with as complete certainty.

Conversely, to compute maximum growth temperatures from minimum temperatures of destruction for the three enzymes, indo-phenol oxidase (4), catalase (5), and succino-dehydrogenase (6), use is made of equations of regression of  $X$  on  $Y$ , (dotted lines on graphs).

$$X = -12.5 + 1.18Y \quad (4)$$

$$X = -6.6 + 1.07Y \quad (5)$$

$$X = -32.8 + 1.65Y \quad (6)$$

When the value for  $Y$ , 48°C. (minimum temperature of destruction of indo-phenol oxidase of the 21 strains of *B. cereus*) is substituted in equation (4) and the value of  $X$  computed, the answer differs from the observed value by -1.0°C. Differences found for the means of the groups of all three enzymes are recorded in table 5.

In only three instances (two strains) were the differences quite large; in many they were within 3 degrees. The majority of differences in degrees were of a magnitude smaller than the range the weighted mean of which is the maximum growth temperature.

On the whole, the inconsiderable differences shown in tables 4 and 5 yield another measure of the accuracy of the hypothesis that a relationship exists between the two variables, maximum growth temperatures and minimum temperatures of destruction of enzymes. Close agreement in so many instances was contributory evidence that the thermolabile nature of certain respiratory enzymes may explain failure of certain organisms to grow above a definite temperature range.

Aside from this principle use of the regression equations to show the degree of relationship between the two factors, the labor involved in measuring and computing minimum temperatures of destruction of enzymes, or maximum growth temperatures of bacteria, would be materially reduced if it could be demonstrated that one enzyme would serve as well as another,

TABLE 5  
*Relations Between Observed and Computed Values of the Maximum Growth Temperatures of Bacteria*

PROBABLE CLASSIFICATION	INDO-PHENOL OXIDASE TEMPERATURE OF DE- STRUCTION Y	MAXIMUM GROWTH TEMPERATURE X = -12.5 + 1.18Y			CATALASE TEMPERATURE OF DESTRUCTION Y	MAXIMUM GROWTH TEMPERATURE X = -0.64 + 1.07Y			SUCCINO-DE- HYDROGENASE TEMPER- ATURE OF DESTRUCTION Y	MAXIMUM GROWTH TEMPERATURE X = -32.3 + 1.03Y		
		X Ob- served	X Com- puted	Differ- ence		X Ob- served	X Com- puted	Differ- ence		X Ob- served	X Com- puted	Differ- ence
	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.
<i>Bacillus mycoides</i> .....	41	40	36	-4	41	40	37	-3	40	40	34	-6
<i>B. praenitens</i> .....	44	40	39	-1	40	40	36	-4	40	40	34	-6
Unidentified.....	52	42	48	6	54	42	51	9	49	42	48	6
<i>B. simplex</i> .....	55	43	52	9	52	43	49	6	40	43	34	-9
<i>B. cereus</i> .....	48	45	44	-1	45	45	42	-3	50	45	50	5
<i>B. megatherium</i> .....	48	46	44	-2	50	46	47	1	47	46	45	-1
<i>B. tumescens</i> .....	55	46	52	6	46	46	43	-3	46	46	43	-3
<i>B. alvei</i> .....	51	46	47	1	50	46	47	1	53	46	55	9
<i>B. pelastis</i> .....	47	46	43	-3	51	46	48	2	45	46	42	-4
Unidentified.....	50	48	47	-1	54	48	51	3	57	48	61	13
<i>B. niger</i> .....	65	49	64	15	65	49	63	14	50	49	50	1
Unidentified.....	58	51	56	5	55	51	52	1	52	51	53	2
Unidentified.....	57	52	55	3	57	52	54	2	53	52	55	3
<i>B. subtilis</i> .....	60	54	58	4	56	54	53	-1	51	54	52	-2
<i>B. vulgaris</i> .....	56	55	54	-1	58	55	53	-2	50	55	50	-5
Unidentified.....	56	57	54	-3	64	57	62	5	55	57	58	1
<i>B. mesentericus-fuscus</i> .....	60	58	58	0	63	58	61	3	53	58	55	-3
Thermophiles.....	65	76	64	-12	67	76	65	-11	59	76	65	-11

or as well as all three together, for estimation. This can be determined by a comparison of standard errors of estimate.

For the equation of regression fitted to these data, which shows the effect of minimum temperature of destruction of the three enzymes, collectively, upon the maximum growth temperature of bacteria,

$$X_1 = -19.61 + 1.18X_2 + 0.012X_3 + 0.133X_4 \quad (7)$$

where  $X_1$  is the maximum growth temperature of bacteria, and  $X_2$ ,  $X_3$  and  $X_4$  are the minimum temperatures of destruction of indo-phenol oxidase, catalase, and succino-dehydrogenase, respectively, it could be shown that

$$S_{1.234} = \pm 5.022^\circ\text{C}.$$

This would indicate that estimates made by use of the equation of regression would not in 68 out of 100 instances differ from observed values by more than  $\pm 5.0^\circ\text{C}$ . Since it was shown that values for  $S_v$  for indo-phenol oxidase, catalase, and succino-dehydrogenase were  $\pm 4.0^\circ\text{C}$ ., or less, it was evident that any one of the enzymes alone was a better measure and represented the relationship more accurately than did all three collectively. The enzyme chosen should have the highest coefficient of correlation,  $r$ . Thus, either indo-phenol oxidase or catalase could be used to the best advantage in estimating an unknown maximum growth temperature of a given species of the genus *Bacillus*.

The determination of maximum growth temperature requires large amounts of media and time, whereas enzyme destruction temperature determinations are relatively simple. Preliminary determinations can be made and approximate values arrived at easily by the use of equations 4, 5 and 6. However, the final accurate value for maximum growth temperatures is found better by the more laborious method explained earlier in this paper.

Advantage of mathematical treatment in obtaining a true evaluation of data, particularly where many individual instances or trials are considered, is quite apparent, since the single value, 0.8466.  $R_{1.234}$ , was found to represent actual general degrees of

relationship existing between minimum temperatures of destruction of three thermolabile enzymes, indo-phenol oxidase, catalase and succino-dehydrogenase, although at a sacrifice of detail. A table containing 1,872 items would be required to illustrate this same degree of relationship, if original protocols (table 2) were included.

Equations of estimation permit a comparison of observed and computed values. The nearer these are to identity, the more certain is the conclusion that this relationship can be truly expressed in mathematical terms.

#### DISCUSSION

It should be pointed out that these statistical methods do not establish causal relationships; yet, a definite relationship of a linear nature does apparently exist, and similarity of the maximum growth temperatures and corresponding minimum temperatures of destruction of various enzymes in so many trials is highly suggestive of the possible rôle the latter may have in the limitation of bacterial growth at higher temperatures.

It is at least probable that, if one or more of these enzymes is destroyed, either the organism must cease to respire and hence fail to grow, or another mechanism of respiration must be substituted in its place. Experimental data reported here indicate that when respiratory enzymes are destroyed growth ceases. Actual destruction of enzymes, but not cells, was effected. Suspensions heated at temperatures destructive for enzymes gave negative tests at regular intervals over a period of a month, when held at temperatures too low to permit growth of cells.

The following question naturally presents itself: Does there exist a multiplicity of the three enzymes, indo-phenol oxidase, catalase and succino-dehydrogenase, each possessing different maximum temperatures of destruction, or do certain cells possess ability to protect their respiratory enzyme complexes against destructive effects of increased temperature to a greater degree than do others?

Failure of other workers to isolate in pure state different types of pepsin, trypsin and other common enzymes, in respect to

their thermolabile properties, although they are obtained from different species of living organisms, does not lend support to the theory of a multiplicity of enzymes.

Virtanen suggested that spores are more resistant to heat than vegetative cells, because a firmer enzyme-protein union is present in spores. By analogy, resistance of thermophiles may perhaps be explained by a firmer enzyme-protein union than is found in mesophilic organisms. This would obviate the need of multiplicity of enzymes, to explain results. Until cell-free purified preparations of these three respiratory enzymes are obtained from various microorganisms having different maximum growth temperatures, the question must remain unanswered, in so far as the enzymes studied here are concerned.

Demonstration of actual destruction of respiratory enzymes, especially in the light of the above theory of an enzyme-protein union of greater or lesser completeness, may serve to emphasize the possible rôle of respiratory enzymes in the regulation of the upper limits of temperature of growth tolerated by individual strains or types of bacteria.

#### BRIEF SUMMARY

Results obtained from a study of three different thermolabile respiratory enzymes of 104 different strains of organisms (representing 18 members of the genus *Bacillus*, and 9 known thermophiles) are summarized as follows:

1. Weighted mean maximum growth temperatures were computed. These do not constitute clearly cut dividing lines, but may be defined as weighted means of the range including both positive and negative results within a series of multiplicate trials.

2. Physical state of the medium, solid or liquid, exerts no apparent effect upon upper limits of growth of the bacteria studied.

3. Significant differences were shown between maximum growth temperatures of the various groups.

4. A thermostable peroxidase was found in each species studied. No evidence of hydrogen peroxide was detected.



5. Determinations of the minimum temperatures of destruction of indo-phenol oxidase, catalase and succino-dehydrogenase were made and compared statistically with maximum growth temperatures of bacteria. A high degree of correlation was found for each enzyme individually and for all three collectively.

6. The equation derived for indo-phenol oxidase gave a more nearly accurate computed value than did those of either catalase or succino-dehydrogenase singly, or than did all three collectively.

7. The observations reported here suggest that to a marked degree the maximum growth temperatures of bacteria may bear a definite relationship to the minimum temperatures of destruction of respiratory enzymes.

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# MORPHOLOGICAL AND CULTURAL STUDIES OF THE GENUS *FUSIFORMIS*

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Although the anaerobic, non-sporulating, pointed bacilli of the mouth, which are now grouped in the genus *Fusiformis*, have been cultivated since 1898 (Veillon and Zuber), much confusion still exists concerning their properties and relationships. The literature dealing with this group of bacteria is large, but it consists mainly of efforts to establish their pathological significance on the basis of occurrence. Only a few systematic studies have been attempted, and the findings of these investigations have been contradictory.

Krumwiede and Pratt (1913) divided 15 strains of *Fusiformis* into 2 classes by means of the fermentation of sucrose. Knorr (1922) created 3 morphological groups on the basis of his study of 4 strains. He employed *Fusobacterium* as the generic name. This classification was used later in "Determinative Bacteriology" by Lehmann-Neumann-Breed (1931). Varney (1927) divided 18 strains into 4 types and 2 subtypes on morphological and serological grounds. Pratt (1927), repeating her earlier work, found 2 morphological groups and concluded that serological procedures had little value in the classification of *Fusiformis*. As a result of his investigations, Smith (1932) was led to accept 3 morphological types. Bibby and Knighton (1933) reached the same conclusions. Like Pratt, they were unsuccessful in their attempts to employ serological methods. The most extensive study of *Fusiformis* was made by Slanetz and Rettger (1933). They studied 53 strains isolated from many sources and were

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able to divide them into 4 types apparently on the basis of morphological and fermentative properties. Their types I and II fermented glucose only; type III fermented glucose and sucrose; and type IV, glucose, sucrose and lactose. Types I and II and some of the strains of type III produced indol. Agglutination tests were unsatisfactory. They present a comprehensive review of the literature.

It is evident from a survey of the literature that information concerning the fusiform bacteria of the mouth is, for the most part, vague and contradictory. Consequently, it seemed to be important to study these bacteria in an orderly manner, with a view to determining the number of well-defined varieties that could be grown in pure culture, and to determining the characteristics of each variety as a basis for systematic arrangement. Such a study has been made of 104 strains of fusiform bacteria isolated from the mouth and grown in pure culture. The present paper presents the findings of the investigation, and suggests a simple, tentative classification.

#### METHODS AND MATERIALS

*Culture media:* No single culture medium proved to be ideal for the primary isolation of all species of fusiform bacteria. Two species were most easily isolated on 5-per-cent rabbit-blood agar, containing gentian violet, 1 part in 5,000. A third species was more conveniently obtained on fresh 10-per-cent potato-extract agar (Slanetz and Rettger, 1933), containing gentian violet, 1 part in 10,000. This small amount of dye was indispensable for routine isolations because without it other bacteria overgrew the plates. For carrying all of the types of *Fusiformis* in subculture, 5-per-cent rabbit-blood agar was employed. Other media included peptone water, with and without added tryptophane, nitrate broth, peptone iron agar (Bacto), Douglas' agar, Douglas' broth with ascitic fluid, plain agar, Sabouraud's agar, and gelatin (plain, and enriched with 10 per cent ascitic fluid). Fermentation tests were made on 15-per-cent ascitic fluid agar, containing Andrade's indicator and 1 per cent of the test carbohydrate; glucose, levulose, sucrose, lactose, maltose, inulin and mannitol

were employed. Tests were observed for 14 days. Negative tests were always repeated. Douglas' broth was used in tests for indol production. Acid production was measured in Douglas' broth containing glucose, as already reported (Hine, 1935). Observations for motility were made by darkfield examination and by the use of semi-solid media (Hine, 1935).

*Technique for primary isolation from the mouth:* For the primary isolation of fusiform bacteria from the oral cavity, material from the chosen site was collected on suitable dental instruments and suspended in a few drops of Douglas' broth. It was then cultured, as already indicated, on gentian violet, potato-extract agar and gentian-violet rabbit-blood agar. (The dye apparently does not inhibit *Fusiformis*. It should be noted that, as the gentian violet does not kill, but merely inhibits certain bacteria, it is essential to streak the picked colony on a dye-free medium, in order to be sure that no other organisms are present.) Incubation was carried out in Fildes-McIntosh jars at 37°C. from 3 to 7 days.

*Source of strains:* The strains of *Fusiformis* were isolated from gingival crevices in "normal" mouths, from cases with deep caries, inflamed areas around crowns and fillings, pyorrhetic pockets, Vincent's infection, and from the mouths of 3 monkeys and 2 rabbits. One hundred and four strains were isolated and grown in pure culture.

*Methods for obtaining anaerobiosis:* To obtain consistently good surface colonies of bacteria belonging to the genus *Fusiformis*, a technique which will provide excellent conditions for anaerobic growth must be employed. In our hands, the use of Brown's (1922) modification of the Fildes-McIntosh jar has proved to be most satisfactory. Some of the stock cultures were kept in chopped meat media covered with paraffin.

*Methods for determining resistance to environmental change:* The resistance of the anaerobic fusiform organisms to air was tested by exposing 72-hour cultures in Douglas' broth, enriched with 10-per-cent ascitic fluid, as follows: 3 cc. of culture were pipetted into sterile Petri dishes and stored at 20°C. After intervals of 1, 1.5, 2, 4, 6, 8 and 24 hours, a loopful was streaked

on rabbit-blood agar, and incubated anaerobically to determine viability. Resistance to heat was tested by pipetting 3 cc. of a 72-hour culture into sterile test tubes, which were immersed in water baths at 50, 52, 56 and 60°C. After intervals of 2, 5, 10 and 15 minutes, samples were removed and the viability of the bacteria tested.

#### FINDINGS

As a result of the detailed study of 67 strains of *Fusiformis*, it has been found that 3 morphologically distinct varieties can be isolated with ease from almost every adult mouth. The average dimensions of organisms of each group are sufficiently constant to make a morphological grouping useful. Since the colony form for each of the morphological groups is characteristic and sufficiently distinctive to permit easy recognition, it has been found convenient to regard these 3 morphological varieties as 3 "species." Such a procedure is open to question, for the fusiform bacteria are notoriously pleomorphic. However, as this grouping was supported by colony form and certain growth characteristics, it was of practical value to consider these "species" separately. Although our findings are not identical with those given by Bergey (1934) in his "Determinative Bacteriology," it is convenient, tentatively, to use his names for our 3 "species." A detailed description of each "species" follows.

*Fusiformis nucleatus*: The group to which the species name *F. nucleatus* is applied is composed of straight, pointed, Gram-negative rods showing usually 1 or 2 deep-staining granules. The organisms are from 3 to 5 $\mu$  in length and from 0.5 to 0.7 $\mu$  in thickness. They often appear in pairs and tend to clump in a characteristic manner (plate 1, figs. 1 and 2).

On 5-per-cent rabbit-blood agar, the colonies of this "species" are round, convex, greyish-white, entire edged, from 0.5 to 1 mm. in diameter, with a smooth and glistening surface. The growth emulsifies readily and is not adherent to the medium. The internal structure of the colony is distinctive, for it appears to be filled with many small white flecks suspended in a light grey medium. With light held at an angle of 45° to the line of vision,

this distinctive feature of the colony may be observed to best advantage (plate 1, figs. 3 and 4).

In Douglas' broth a good growth occurs at the bottom of the tube, if incubation has been carried out in an anaerobic jar. On shaking, the broth becomes uniformly turbid.

Much variation in saccharolytic activity was noted among the 24 strains studied. The results of the fermentation tests are recorded in table 1. Four of the strains refused to grow on ascitic fluid agar although they grew well on rabbit-blood agar. Indol was produced by all of the strains and the pH of the test solution was slightly depressed. None of the strains grew in peptone

TABLE 1

*Fusiformis nucleatus*: Biochemical characteristics of 24 strains

	NUMBER OF STRAINS					
	4	2	6	6	5	1
Glucose.....	0	+	+	+	+	+
Levulose.....	0	-	+	+	+	±
Sucrose.....	0	-	-	+	+	-
Lactose.....	0	-	-	-	+	+
Maltose.....	0	-	-	-	-	±
Inulin.....	0	-	-	-	-	-
Mannitol.....	0	-	-	-	-	-
Indol.....	+	+	+	+	+	+
Average pH.....	6.4	5.5	6.3	6.2	5.2	

+ = fermentation; - = no fermentation; 0 = no growth.

water, nitrate broth, peptone iron agar (Bacto), Sabouraud's agar, or gelatin, but scanty growth occurred on plain agar; good growth resulted in chopped meat media in which some of the strains survived for as long as 18 months. Prolonged cultivation sometimes produced rough granular colonies, often presenting a "poached egg" appearance (plate 1, figs. 5 and 6). No motility was observed in cultures.

Pure cultures of this "species" survived exposure to air for 6 hours on the average. The 6 strains tested survived temperatures of 56°C. for 15 minutes, but at 60°C., killing occurred within 10 minutes.



*Fusiformis polymorphus*: The group to which the species name *F. polymorphus* is applied is composed of straight, slightly pointed, Gram-negative rods, occasionally showing a granular structure in old cultures. The organisms are normally from 6 to 10 $\mu$  in length and from 0.4 to 0.6 $\mu$  in thickness, but they may form long filaments, 250 $\mu$  in length. Young cultures may show many long, flexible filaments that often become wavy or curled (plate 2, figs. 1 and 2).

On 5-per-cent rabbit-blood agar, the colonies of this "species" are greyish-white, round, convex, entire edged from 1 to 2 mm. in diameter, with a smooth and glistening surface. When mag-

TABLE 2  
*Fusiformis polymorphus*: Biochemical characteristics of 16 strains

	NUMBER OF STRAINS			
	2	4	5	5
Glucose.....	—	+	+	+
Levulose.....	—	+	+	+
Sucrose.....	—	—	+	+
Lactose.....	—	—	—	—
Maltose.....	—	—	—	±
Inulin.....	—	—	—	—
Mannitol.....	—	—	—	—
Indol.....	+	+	+	+
Average pH.....	7.4	6.0	6.1	6.2

nified, they appear to be composed of a mass of thin white lines suspended in a water-clear medium. The colonies are usually larger than those of *F. nucleatus* and the internal characteristics are more distinct. When light falls at an angle of 45° to the line of vision, this type of colony is easily located (plate 2, figs. 3, 4 and 5). Growth in broth is similar to that of *F. nucleatus*.

The biochemical characteristics of 16 strains of *F. polymorphus* are shown in table 2. Marked variation in saccharolytic activity by these morphologically indistinguishable strains was noted. They all grew well on ascitic agar. All of the strains produced indol and a slight amount of acid. None grew in peptone water,

nitrate broth, peptone iron agar (Bacto), Sabouraud's agar or gelatin. Slight growth appeared on plain agar. All grew well in chopped meat media in which some of the strains survived for 15 months. Prolonged cultivation sometimes yielded colonies with an irregular edge, rough surface and amorphous internal structure (plate 2, fig. 6). The cells from such colonies were atypical, showing many odd-shaped forms. No motility was observed in cultures.

Pure cultures survived exposure to air for 4 hours on the average. When mixed with an aerobe, survival was extended to 24 hours. The 4 strains tested survived 50°C. for 15 minutes, 52°C. for 10 minutes and 56°C. for 5 minutes.

*Fusiformis dentium*: The group to which the species name *F. dentium* is applied is composed of the largest culturable type of fusiform bacterium. The organisms are straight, or slightly curved, sharply pointed, Gram-negative rods. In direct smears from the mouth or in the first culture, the bacilli occasionally show Gram-positive granules. The cells often occur in a tandem arrangement (plate 3, figs. 1 and 2).

The colonies of this "species" are circular, from 1 to 3 mm. in diameter, shaped like a low cone, with a surface which resembles hammered copper or which may be wrinkled and pitted. The colonies usually have a fairly regular edge and are easy to pick and emulsify. Identification is easy if the plate is tilted at an angle of 45° to the line of vision with the light striking the plate at an angle of 90° (plate 3, figs. 4 and 5). Under ordinary illumination, the growth is practically invisible. No marked changes in colony type were noted, even after prolonged cultivation.

The biochemical characteristics of 27 strains are shown in table 3. Two strains failed to grow on ascitic fluid medium and 2 others on lactose ascitic fluid agar. All of the strains failed to produce indol. The pH was shifted to slightly below 5. None of the strains grew in peptone water, nitrate broth, peptone iron agar (Bacto), Sabouraud's agar, gelatin or plain agar. Growth occurred in chopped meat media in which some strains survived for 3 months. No motility was observed in cultures.

Pure cultures survived exposure to air for 1 hour and, when mixed with an aerobe, survived for 4 hours. The 4 strains tested survived 60°C. for 2 minutes, but not for 5 minutes.

*Observations on films from the mouth:* The examination of films prepared directly from the mouth revealed that organisms morphologically resembling the cultivated species were present in great numbers in adult mouths. In addition, a fourth morphological variety was often found, especially in instances of marked inflammation. This organism was sharply pointed, granular, curved, from 10 to 20  $\mu$  in length and about 1.2  $\mu$  in thickness. This form has not been duplicated in culture, but

TABLE 3  
*Fusiformis dentium*: Biochemical characteristics of 27 strains

	NUMBER OF STRAINS				
	2	11	10	2	2
Glucose.....	0	+	+	+	+
Levulose.....	0	+	+	+	+
Sucrose.....	0	+	+	+	+
Lactose.....	0	+	—	±	0
Maltose.....	0	+	+	+	+
Inulin.....	0	—	—	—	—
Mannitol.....	0	—	—	—	—
Indol.....	—	—	—	—	—
Average pH.....		4.8	4.8	4.9	

resembles the granular forms of *F. dentium* (plate 3, fig. 3). Whether this uncultivated fusiform organism is a distinct species, or a variant, of *F. dentium*, has not been determined.

Correlated studies of the spirochetes seen in smears from the mouth and the cultured fusiform organisms failed to support the possibility of a generic relationship between these types of microorganisms. In over 60 per cent of the stained film preparations of *F. dentium*, definite spiral forms were found. Similarly, many preparations of *F. polymorphus* showed wavy, filamentous forms. However, these spiral forms bore but a superficial resemblance to oral spirochetes, and their size and staining reactions made it apparent that the wavy forms were all *Fusiformis*.

Furthermore, when examined under the darkfield microscope, they never exhibited the motility or flexibility which characterizes the oral spirochetes.

#### DISCUSSION

On the basis of the findings presented in this paper, it appears that the fusiform bacteria of the mouth include 3 or possibly 4 morphological "species." Three "species" of *Fusiformis* have been readily cultivated from almost every adult mouth. In no instance has one morphological "species" changed into another. Some of the strains have been carried for 18 months through many culture generations without loss of the original characteristics. It is felt, therefore, that the groups may be regarded with some justification as distinct "species." Fermentation tests have indicated that each of the 3 cultivated "species" can be divided into varieties, but careful checking of these "species" and varieties has failed to show any correlation between morphology, colony form or biochemical reaction and the source of the strains. Consequently, until the significance of the various "species" is established, it seems of little value to subdivide them.

It has been impossible to correlate all of the published classifications of *Fusiformis*, for the information furnished has not usually been of the type to make comparisons feasible. It is probable, however, that the "species" which we have designated as *F. nucleatus* includes Varney's type III, Smith's type III, Slanetz and Rettger's type I and Knorr's *F. nucleatus*. Similarly, our *F. polymorphus* probably includes Varney's types I and III, Smith's type II, Slanetz and Rettger's type II and Knorr's *F. polymorphus*, and our *F. dentium* probably includes Varney's type IV, Smith's type I, Slanetz and Rettger's types III and IV and Knorr's *F. plaut-vincenti*. The probability of this grouping being correct is supported by the close agreement in size and growth characteristics reported by these authors. This is especially significant, when the different growth conditions used by each are considered.

It has been our experience that strains of *F. nucleatus* are the

easiest to maintain in pure culture and the most resistant to environmental change. Strains of *F. polymorphus* are almost as easily grown, but strains of *F. dentium* must be handled carefully, because exposure to air for short periods, or mild heating kills the organisms. These facts explain, undoubtedly, why most of the earlier reports of successful cultivation of oral fusiform bacteria deal with the first and second "species" only.

Published reports of fermentation tests are few and usually concern only a few strains. An exception is the report of Slanetz and Rettger (1933), who worked with 53 strains. In repeated tests, using the media they recommended and, in addition, ascitic fluid carbohydrate agar, we were unable to duplicate their findings. It is conceivable that the greater saccharolytic activity of our strains of *Fusiformis* might be related to differences in the anaerobic conditions under which the tests were made.

It is apparent from the literature and from our observations that most of the suggested classifications of the fusiform bacteria have little value and that the majority of them are based on incomplete studies. The only proposed classifications, which embrace all the types we have observed, are those of Slanetz and Rettger (1933), based on fermentation reactions and of Bergey (1934), based on morphological differences. Since our studies and those of Bibby and Knighton (1937) have failed to show the correlation of colony form, morphology and biochemical properties described by Slanetz and Rettger (1933), we cannot accept their classification. Although Bergey's classification does not list all the features observed by us, it appears to be the most useful basis for differentiation of the fusiform bacteria. We suggest, therefore, the employment of Bergey's division of *Fusiformis* into the "species" *F. nucleatus*, *F. polymorphus* and *F. dentium* as a useful working basis for further work.

#### SUMMARY

One hundred and four strains of fusiform bacteria have been isolated from the mouth and studied in pure culture. The results of these observations are presented in detail. On the basis of these studies, it appears useful to divide, tentatively, the genus

*Fusiformis* into 3 "species," *Fusiformis nucleatus*, *Fusiformis polymorphus* and *Fusiformis dentium*. These are the names used by Bergey (1934), and although our findings are not in complete agreement with those listed by him, our differentiation into "species" is based on well-defined morphological and cultural differences. Certain biochemical characteristics of each "species" are discussed. While fermentation tests indicate that each "species" can be divided into varieties, we feel that further subdivision of *Fusiformis* at present has no practical value.

The authors take pleasure in thanking Dr. B. G. Bibby for his advice and aid. They are indebted to Mrs. R. P. Ball and Mr. M. Orser for taking the photographs.

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## PLATE 1

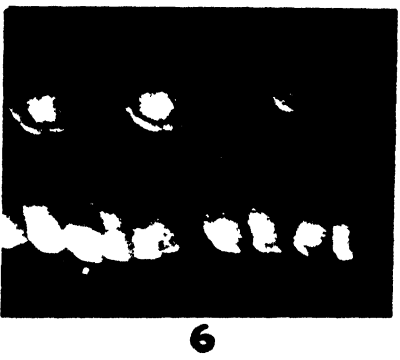
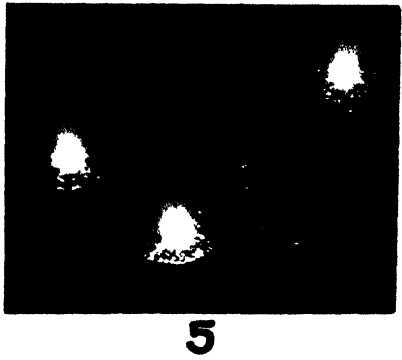
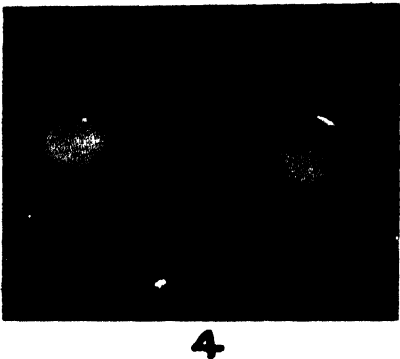
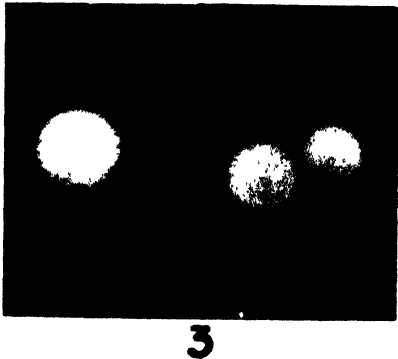
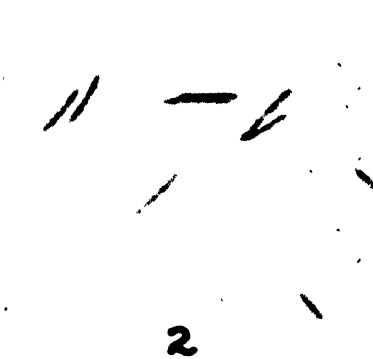
FIG. 1. *Fusiformis nucleatus*. Pure cultures on 5 per cent rabbit-blood agar, third transfer, 3 days old. Stained with gentian violet.  $\times 1000$ .

FIG. 2. *Fusiformis nucleatus*. Pure culture in Douglas' broth, 3 days old. Gentian violet stain.  $\times 1400$ .

FIGS. 3 and 4. *Fusiformis nucleatus*. Colony on 5 per cent rabbit-blood agar, with light striking plate at angle of  $45^\circ$ .  $\times 14$ .

FIG. 5. *Fusiformis nucleatus*. Rough colonies which appeared after the sixth transfer of strain 76 on 5 per cent rabbit-blood agar.  $\times 14$ .

FIG. 6. *Fusiformis nucleatus*. "Poached egg" appearance of colonies which appeared after the sixth transfer of strain 79 on 5 per cent rabbit-blood agar.  $\times 14$ .



(Maynard K. Hine and George Packer Bertz - Studies of Genus *Fusiformis*)



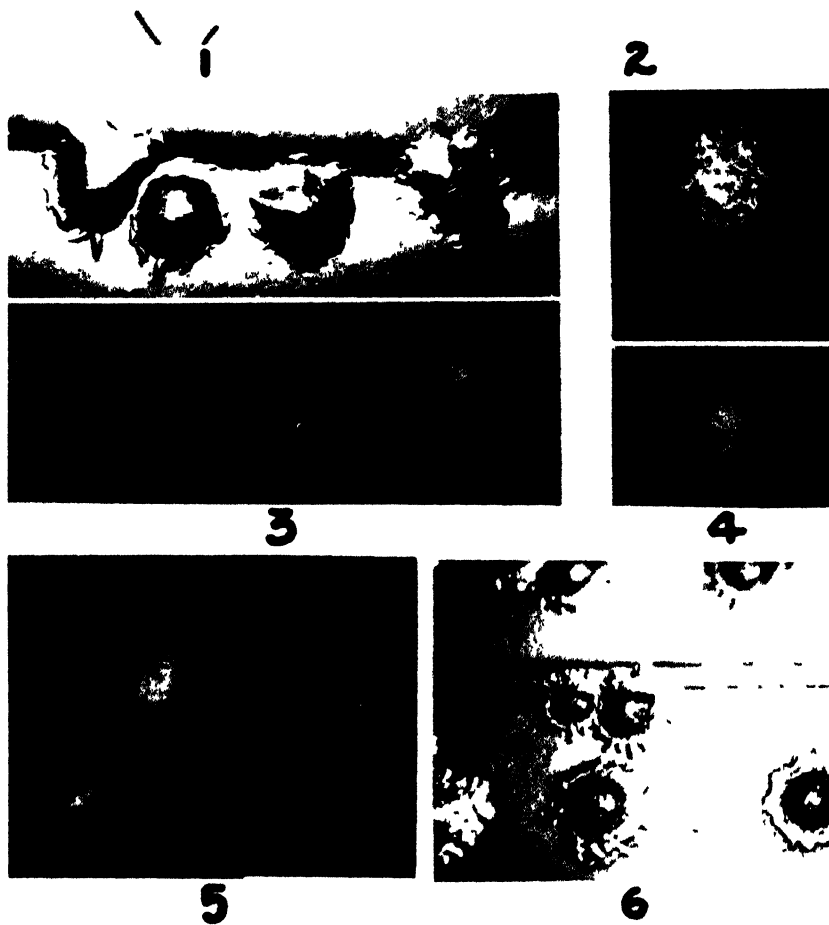
## PLATE 2

FIGS. 1 and 2 *Fusiformis polymorphus*. Pure culture in Douglas' broth, 3 days old. Note rounded ends and tendency to form filaments. Stained with gentian violet.  $\times 1000$

FIG. 3. *Fusiformis polymorphus*. Four typical colonies on 5 per cent rabbit-blood agar, 3 days old. Upper photograph was taken with plate tilted to an angle of  $45^\circ$  with line of vision and with the light striking the plate at an angle of  $90^\circ$ . The lower photograph was taken with the plate held at an angle of  $90^\circ$  to the line of vision and with the light striking the plate at an angle of  $45^\circ$ .  $\times 14$ .

FIGS. 4 and 5 *Fusiformis polymorphus*. Typical colonies on 5 per cent rabbit-blood agar.  $\times 14$ .

FIG. 6. *Fusiformis polymorphus*. Rough colonies which appeared after the sixth transfer of strain 59 on 5 per cent rabbit-blood agar.  $\times 14$ .



(Maynard K. Hine and George Packer Berry: Studies of Genus *Fusiformis*)

## PLATE 3

FIG. 1 *Fusiformis dentium*. Pure culture in Douglas' broth, 3 days old. Note sharply pointed ends. Stained with gentian violet  $\times 1000$ .

FIG. 2. *Fusiformis dentium*. Pure culture in Douglas' broth, 3 days old after 8 hours exposure to air. Stained with gentian violet  $\times 1000$ .

FIG. 3. *Fusiformis dentium* (?). Direct film from the mouth, showing organisms not duplicated in culture. Tunneliff modification of Gram's stain  $\times 1000$ .

FIGS. 4 and 5. *Fusiformis dentium*. Typical colonies on 5 per cent rabbit-blood agar.  $\times 14$ .



(Maynard K. Hine and George Packer Berry: Studies of Genus *Fusiformis*)



# THE FUSOBACTERIUM GENUS

## I. BIOCHEMICAL AND SEROLOGICAL CLASSIFICATION

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Fusiform bacilli are normally present in human mouths and throats, and on the external genitalia; also in the oral cavities of various species of animals. They have been observed, furthermore, in a variety of human pathological conditions. In spite of their frequent occurrence in nature, there has been little progress in the classification of these organisms since Leiner (1907) first observed that there was more than one cultural type. This situation may be ascribed largely to the difficulty of obtaining satisfactory growth upon solid media. Most of the attempts at classification have been limited to a very large extent to morphological observations. Furthermore, it has been held generally that all spindle-shaped organism are members of the fusiform genus. The resulting confusion is evidenced by the fact that the relevant literature contains many contradictory reports concerning staining properties, motility, oxygen requirements and biochemical reactions.

The first systematic study was that of Krumwiede and Pratt (1913), who divided fifteen strains into two groups, upon their ability to ferment sucrose. Knorr (1922) established three morphological types among his strains, as did Smith (1932), by the use of the dark-field illumination technique. In 1927 Varney published a detailed description of eighteen cultures from normal and abnormal mouths. Four types were estab-

<sup>1</sup> This paper covers part of a dissertation submitted to the Graduate School of Yale University in partial fulfilment of requirements for the degree of Doctor of Philosophy.

lished, two by serological methods, and two by morphological studies. Biochemical observations were not reported.

Slanetz and Rettger (1933) made a significant advance in the study of the fusobacteria by devising a potato medium upon which abundant growth could be obtained. A morphological and biochemical study of fifty-three strains revealed four types. Types I and II were indistinguishable biochemically, both fermenting glucose and producing indol, but were separated by colony appearance and cell size. Type III was larger than I and II, and fermented sucrose as well as glucose. Indol formation was irregular. Type IV was a large bacillus which fermented lactose as well as glucose and sucrose, but did not form indol. The agglutination test was considered as unsuitable for the purpose of classification. Quite recently Bachmann and Gregor (1936) divided nine strains into two groups by means of agglutination and complement fixation tests. No biochemical reactions were reported.

Unfortunately, it is not possible to compare the types proposed by the various investigators since, in no instance, has one group of cultures been successfully studied by both biochemical and serological methods. The present report deals with a morphological, cultural, biochemical and serological study of eighty strains originating from a variety of sources. All of the cultures were Gram-negative, non-motile, non-sporulating and, when isolated, strictly anaerobic.

#### METHODS

*Media employed.* Slanetz and Rettger found gentian violet to be a valuable aid in primary isolation, on account of its favorable selective action. The medium which proved most satisfactory during the present investigation was the same as that of Slanetz and Rettger, except that it contained glucose and cysteine. It has the following composition:

Proteose peptone.....	1.0 per cent
Liebig's meat extract.....	0.3 per cent
Cysteine hydrochloride.....	0.05 per cent
Glucose .. . . .	0.1 per cent

Potato extract (aqueous)	10 0	per cent
Agar	2 0	per cent
Distilled water		q.s.
Adjusted to pH 7.6		

For making primary isolations gentian violet was added in a final concentration of 1:20,000.

Stock cultures were carried in a semi-solid agar (0.3 per cent) having the above composition, aside from the agar. For routine purposes the same medium without the agar was used. All media were sterilized at 15 pounds pressure for 15 minutes, cooled quickly, and inoculated immediately, when this was possible. When media were held overnight they were stored in an anaerobic jar.

*Method of isolation.* Swabs containing the original material were streaked on the gentian violet agar. When the inoculum came from human mouths, members of the *Fusobacterium* genus were usually obtained directly in pure culture. Material from animal sources, however, frequently contained other organisms to such an extent as to overgrow the more delicate fusiform bacilli; this made it necessary to dilute the original material. In making isolations from cases of pulmonary infection precaution was exercised to avoid contamination with mouth flora. Sputum as such was diluted with two volumes of sterile saline solution and centrifuged lightly. After removing the supernatant fluid, the washed residue was re-suspended in an equal volume of saline solution, shaken vigorously and whirled at high speed for ten minutes. The supernatant liquid yielded *Fusobacterium*, frequently accompanied by minute cocci.

The gentian violet agar plates were incubated anaerobically for two days at 37°C., at the end of which period the *Fusobacterium* colonies could be recognized by their deep violet color and characteristic ground-glass appearance when viewed with the hand lens. Subcultures from these colonies were usually pure.

*Anaerobic technique.* The method for securing strictly anaerobic conditions was that described by Weiss and Spaulding (1937). The procedure consists in evacuating a glass jar (Hempel desiccator) which is connected with a system containing a Cenco Hyvac pump, mercury manometer and hydrogen tank. A small



flat porcelain dish containing a catalyst (shredded palladinized asbestos) is placed conveniently in each jar, the ground surfaces of which are smeared lightly with cello-seal. When the jar has been evacuated to the negative pressure desired, the pump is disconnected by means of a three-way stopcock. Hydrogen is then passed through the system and into the jar until atmospheric pressure is restored. If the addition of a small amount of carbon

TABLE 1  
*Sources of strains*

	TYPES
<i>Human:</i>	
Normal mouths.....	H <sub>1</sub> -H <sub>27</sub> , and H <sub>8</sub> , H <sub>12</sub>
Carious teeth.....	H <sub>28</sub> -H <sub>30</sub>
Fuso-spirochetal infections (mouth and throat) .....	VA <sub>1</sub> -VA <sub>10</sub>
Lung abscess .....	LA <sub>1</sub> -LA <sub>4</sub> , LA <sub>7</sub> , LA <sub>13</sub> , LA <sub>14</sub> -LA <sub>18</sub>
Lung abscess with empyema .....	LA <sub>5</sub> , LA <sub>6</sub> , LA <sub>8</sub> -LA <sub>10</sub>
Bronchiectasis.....	LA <sub>11</sub> , LA <sub>13</sub>
<i>Vagina:</i>	
Normal.....	V <sub>1</sub> , V <sub>2</sub> , V <sub>3</sub>
Cervical laceration .....	V <sub>3</sub>
Purulent discharge.....	V <sub>4</sub>
<i>Animal:</i>	
<i>Chimpanzee:</i>	
Normal mouth.....	Ch <sub>1</sub>
Chronic lung infection .....	CH <sub>2</sub>
Monkey mouths (normal) .....	M <sub>1</sub> -M <sub>4</sub>
Dog mouths (normal).....	D <sub>1</sub> -D <sub>5</sub>
Rabbit mouths (normal).....	R <sub>1</sub> -R <sub>3</sub>
Guinea pig mouths (normal) .....	GP <sub>1</sub> -GP <sub>3</sub>

dioxide is desired, this may be accomplished before introducing the hydrogen, by attaching the carbon dioxide tank to the system.

#### *Sources of strains*

Thirty-two isolations were obtained from so-called normal mouths of twenty-two persons, including two from carious teeth. Ten cultures originated in various cases of mouth infection; for two of these (VA<sub>1</sub> and VA<sub>2</sub>) the writers are indebted to Dr. Ruth Tunnicliff. Sixteen "lung abscess" cultures were derived from

various pulmonary infections. Four of these strains (LA<sub>1</sub> through LA<sub>4</sub>) were kindly supplied by Dr. Charles Weiss. Five vaginal isolations were made from both normal and pathological conditions. Seventeen cultures were obtained from the mouths and throats of animals (see table 1).

#### FREQUENCY AND DISTRIBUTION

*Fusobacterium* colonies appeared on all of the plates streaked with swabs from human mouths. They were less frequently obtained from the throats of animals. All of the swabs from pulmonary as well as mouth and throat infections yielded positive cultures. The material from three out of five normal human vaginæ also contained fusobacteria; on the other hand, thirty swabs obtained during pregnancy were negative. With one exception, washings from the pregnancy cases were distinctly acid, ranging from pH 4.4 to 6.6. Repeated attempts to obtain isolations from human and animal feces were unsuccessful.

#### CLASSIFICATION OF STRAINS

##### *Fermentation studies*

The ability of some of the fusiform bacilli to ferment sucrose and lactose, while others lacked this property, suggested to us that a more comprehensive investigation of the fermentative properties might reveal further differential tests. Therefore, the eighty strains were studied for their ability to produce acid and gas from eighteen fermentable substances. In addition to the common mono- and disaccharides, this list included cellobiose, trehalose, raffinose, inulin, soluble starch, xylose, rhamnose, salicin, glycerol, mannitol, sorbitol and inositol.

Two methods were employed for determining the carbohydrate activity. In the first, the test materials were added aseptically to the basal potato broth medium from autoclaved aqueous solutions of the test substances; in the second, the test solutions were sterilized by filtration. The second procedure is preferable. In each instance the inoculum consisted of 0.1 cc. of a twenty-four-hour potato broth culture. Durham fermentation tubes may be used, since the anaerobic jar holding the tubes

need not be evacuated to the point where the liquid is displaced from the inverted tubes.

The basal medium for the fermentation tests consisted of 1.0 per cent proteose peptone, 0.3 per cent Liebig's meat extract and 0.1 per cent cysteine hydrochloride. Excellent growth occurred in this medium when the carbohydrate present was utilized. The medium must be freshly prepared, however, before use; indeed, satisfactory results cannot be obtained unless the inoculations are made immediately after sterilization.

Sulphonaphthalein indicators could not be added directly to the medium, since they were occasionally destroyed by the organisms. The pH determinations were made by a spot-plate procedure. After twenty-four hours, three days and seven days incubation at 37°C., a bi-convex loopful of the broth culture was added to approximately 0.5 cc. of distilled water containing one drop of a 0.16 per cent solution of the proper indicator. White porcelain spot-plates furnished an excellent background. Although tedious, this method possesses the important advantage of permitting determinations beyond the range of a single indicator. Since the primary division of the authors' cultures is based upon differences in the final pH, this method plays an important rôle in the classification scheme proposed in this report.

Two distinct types of fermentative reactions were observed. One set of cultures (group I) was mildly saccharolytic and produced a relatively weak acidity, the final pH averaging 6.2. The remaining cultures (group II) gave rise to a much greater final acidity (pH 4.6), and in addition were active toward a wider range of carbohydrates. The separation of the two groups on this basis was distinct.

Glucose and levulose were fermented by all of the strains. None of the cultures produced acid from xylose, rhamnose, glycerol, inulin, mannitol, sorbitol and inositol. Galactose was attacked by some members of both groups but with only slight acidity. Maltose and trehalose were acidified by group II, but not by group I. The ability to ferment these two substances constitutes in itself a valuable differential test for the two groups.

Group I is divided into two types. The strains fermenting only glucose and levulose are designated as belonging to type I A; the remaining cultures, all capable of attacking sucrose, constitute type I B. Group II, although it contains no less than nine fermentative patterns, is separated only into a lactose-negative type, II A, and a lactose-positive, type IIB.

TABLE 2  
*Summary of fermentative types*  
Final pH in 1.0 per cent carbohydrate cysteine broth

TYPE	NUM- BER OF STRAINS	GLU- COSE, LEVU- LOSE	SU- CROSE	MAL- TOSE	TREHA- LOSE	SALICIN	CELLO- BIOSE	LACTOSE	RAFFI- NOSE	SOLUBLE STARCH
Group I										
I A	29	ac	0	0	0	0	0	0	0	0
I B	19	ac	ac	0	0	0	0	0	0	0
Intermediate										
	2	AC	AC	0	0	0	0	0	0	0
	3	AC	AC	AC	AC	0 or AC	0 or AC	0	0	0
Group II										
II A	10	AC	AC	AC	AC	0 or AC	0 or AC	0	0	0
II B	1	AC	AC	AC	AC	0	0	AC	0	0
	16	AC	AC	AC	AC	AC	AC	AC	0 or AC	0 or AC

ac indicates final pH between 6.0 and 6.5; AC indicates final pH between 4.4 and 5.2; 0 indicates no detectable acidity.

Two cultures in group I (LA<sub>8</sub> and LA<sub>10</sub>) and three in group II (H<sub>6</sub>, H<sub>9</sub> and H<sub>14</sub>) proved to be atypical in that they possessed some of the biochemical characteristics of the opposite groups. These five cultures were placed together as an intermediate group.

The amount of titratable acidity produced by group II in 1.0 per cent carbohydrate media was found to be approximately twice that of group I, suggesting that group II is able to utilize an intermediate product of carbohydrate metabolism not attacked

by group I. Using the Thunberg technique, as modified by Kendall and Ishikawa (1929), the ability of the two groups to activate pyruvic, acetic and succinic acids was determined. Unfortunately, none of these substances proved of differential value, since pyruvic acid was activated by both groups, and acetic and succinic acids by neither group.

### *Biochemical studies*

*Indol.* With two exceptions, the group I cultures produced indol, as shown by the Böhme-Ehrlich technique. Group II was uniformly negative by this test substance. The potato extract broth medium, without glucose and cysteine, was the most suitable medium.

*Hydrogen sulfide.* Group I formed large amounts of hydrogen sulfide, in the absence of cysteine, while group II blackened lead acetate only in the presence of this agent. Glucose was omitted from the medium. In gelatin or semi-solid agar containing cysteine, the hydrogen-sulfide-positive strains produced a turbid zone at the surface of the medium after a few hours' exposure to the air. This opaque layer contained elemental sulfur.

*Nitrate reduction.* Group I cultures, in general, reduced nitrates, with the liberation of ammonia. Group II, on the other hand, was uniformly negative for nitrite after five days' incubation. The usual naphthylamine-sulphanilic acid test was used for the detection of nitrite, the Thomas test for ammonia production, and zinc dust (ZoBell, 1932) for residual nitrate. The results were most satisfactory in the absence of added glucose and cysteine.

*Hemolysis.* None of our strains was hemolytic for cow's blood, but group I colonies were sometimes surrounded by a greenish area after twenty-four hours' exposure to the air. This "viridans" effect was found to be associated with the production of hydrogen sulfide.

*Liquefaction of gelatin.* Gelatin was never liquefied.

### *Serological studies*

Serological methods have been of comparatively little value in the classification of the fusobacteria. Both Pratt (1927) and

Slanetz and Rettger (1933) emphasized the antigenic individuality of their strains. On the other hand, Varney (1927) established two of his four types by means of the agglutination test, and recently (1936) Bachmann and Gregor distinguished two groups by serological methods.

*Agglutination tests.* Relatively large doses of living organisms were employed for the production of antisera. Twenty-hour agar plate cultures were suspended in physiological salt solution and adjusted to an approximate turbidity of 10, McFarland scale. Five injections, increasing from 1.0 to 5.0 cc., were given at two-day intervals. Only cultures forming a homogeneous suspension were used. After five days following the last injection the titre was usually at the maximum (1:10,000 to 1:20,000). Ten antisera were prepared; however, one serum showed a high prozone reaction with every antigen tested and was discarded. Three of the strains were lethal for rabbits, necessitating the use of formalized antigens.

Performance of successful agglutination tests with most of the *Fusobacterium* strains was accomplished with considerable difficulty, since there was a strong tendency toward spontaneous agglutination. Cells obtained from agar cultures exhibited less non-specific agglutination than did broth cultures. Relatively stable antigens were finally obtained, however, by using twenty-hour meat infusion potato agar cultures suspended in alkaline (pH 8.0) formalized m/20 solution of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ . Readings were made after three hours' incubation at 45°C., and after twenty-four hours' at 37°C. Extreme care was necessary in interpreting the results. Upon standing, many of the larger morphological types (group II) settled out in a manner entirely distinct from auto-agglutination. Viewed casually, this sedimentation bore a striking resemblance to flagellar agglutination, but when the tubes were shaken again a homogeneous suspension resulted. True agglutination of the large group II cells resulted in loose flocculation, while group I underwent typical granular clumping. The discovery that a few of the strains were serum-sensitive necessitated the use of a normal serum control. This phenomenon did not appear to be associated with the presence

of rough variants. Stock antigens stored for several months in the ice box were still satisfactory.

The eighty antigens were tested against the nine antisera; representative reactions are presented in table 3. The strain specificity reported by previous investigators is evident from the sample results shown. It becomes apparent that a classification of the genus based solely upon the simple agglutination test is open to criticism, certainly unless an appreciable number of antisera of each type is employed.

Viewing the agglutination results in a general way, group I is definitely separated from group II, thus confirming the biochemi-

TABLE 3  
*Agglutination reactions*

ANTIGEN		ANTISERUM										Control
		Group I					Group II					
							Type A		Type B	Type A		
		Strain	Type	H <sub>1</sub>	Ch <sub>2</sub>	Ch <sub>1</sub>	LA <sub>4</sub>	H <sub>12</sub>	H <sub>4</sub>	H <sub>11</sub>	LA <sub>3</sub>	
H <sub>30</sub>	I A	640	1,280	1,280	320	160	40	0	0	0	0	
VA <sub>5</sub>	I A	160	10,240	10,240	20	2,560	40	40	0	0	0	
H <sub>22</sub>	I B	160	160	0	160	5,120	0	0	0	0	0	
LA <sub>1</sub>	I B	320	160	80	0	320	0	0	0	0	0	
H <sub>11</sub>	II A	1,280	40	320	0	40	10,240	0	0	0	0	
VA <sub>2</sub>	II A	160	160	20	0	0	2,560	0	0	0	0	
H <sub>29</sub>	II B	0	80	40	20	0	640	20	10,240	160	0	
LA <sub>13</sub>	II B	0	20	0	0	0	40	0	2,560	0	0	

cal differentiation. The intermediate group is divided, four strains showing antigenic relationship to group I, and one to group II. There is some evidence that group II is composed of two distinct serological types corresponding to types II A and II B. Many of the type II B strains, however, possessed agglutinogens for the entire group. The antigenic composition of both groups was investigated further by means of agglutinin absorption and precipitation tests (see table 4).

*Agglutinin-absorption tests.* Reciprocal absorption tests confirmed the presence of group specific antigens. The high degree of strain specificity, however, made interpretation of the results

difficult. Attempts to recognize the presence of definite antigenic types in group I were unsuccessful; in fact, sufficient data were obtained to justify the conclusion that this group possesses a complex antigenic structure. Group II, on the other hand, was divided, as suggested by the simple agglutination tests, into two sub-groups corresponding to types II A and II B. There was a further indication that type II B strains contain agglutinogens for both types II A and II B.

*Precipitin tests.* The successful use of the precipitation technique by Lancefield (1933, 1934) for typing the streptococci, and by Julianelle and Wiegard (1935) for the classification of the

TABLE 4  
*Summary of classification scheme*

	BIOCHEMICAL								SEROLOGICAL			
	Fermentation of			Final pH in car- bohydrate broth	Produc- tion of		Reduc- tion of		Agglutination by antisera against			
	Sucrose	Maltose, Trehalose	Lactose		Ind- ol	H <sub>2</sub> S	NO <sub>2</sub>	NO <sub>3</sub>	Group I		Group II	
									A	B	A	B
Group I, type A . . .	-	-	-	6 2	+	+	+	+	+	+	-	-
Group I, type B . .	+	-	-	6 3	+	+	+	+	+	+	-	-
Intermediate. . .	+	±	-	4 8	+	+	±	±	±	±	±	-
				5 1								
Group II, type A.	+	+	-	4 7	-	-	-	-	-	-	+	-
Group II, type B.	+	+	+	4 6	-	-	-	-	-	-	±	+

± indicates both positive and negative reactions within the group.

staphylococci, suggested the desirability of applying this technique to the fusobacteria. The method of Lancefield in which crude extracts are employed was at first closely followed. When most of these antigens failed to produce any precipitation whatsoever, larger volumes of cells were used, but still with negative results. Believing that much of the desired antigen might have been lost in separating the cells from the medium in which they were grown, cell-free broth antigens were prepared, but again with unsatisfactory results. Finally, the bacterial growths on solid media were washed off and boiled for one hour in M/20 HCl. A study of twenty extracts prepared in this manner re-



sulted in some confusion. The group II antigens reacted only with homologous antisera, but the group I extracts failed to react with any of the antisera. Whereas group II had been divided previously into the two types by the agglutination tests, it appeared by this method to be homogeneous. Five of the antisera were entirely unsatisfactory as precipitating agents. Similar (negative) results were obtained in attempts to isolate a precipitable substance.

The biochemical and serological bases of classification receive some support from the morphological and cultural studies conducted by us. However, since the last two must be regarded as having somewhat limited taxonomic value in themselves, and because they constitute a major part of the work on growth requirements and variation, they are presented in the second of the two papers which constitute this series.

#### RELATION BETWEEN SOURCE AND TYPE

Most investigators have failed to note a correlation between types and sources of strains. On the other hand, Slanetz and Rettger isolated their types II and III from pathological conditions only. In the present study it is noteworthy that all but three of the animal strains belonged to group I.

#### DISCUSSION

If it were not for the presence of the intermediate group, the question might well be raised as to whether groups I and II actually belong to the same genus. Fortunately, the five intermediate strains possess properties characteristic of both groups. As a result, it is possible to trace a gradual transition from the weakly saccharolytic and highly nitrogen-active strains of group I (type A) to the highly fermentative members of group II (type B), which are relatively indifferent to the presence of nitrogen compounds.

The occurrence of essentially different fermentation processes was substantiated further by potentiometric oxidation-reduction determinations made in this laboratory by Dr. R. W. H. Gil-

lespie.<sup>2</sup> The potential time curves clearly demonstrated that the two groups may be distinguished by this method.

The primary separation into two groups was made, not upon the ability of different strains to attack different carbohydrates, but rather upon the widely different final acidities which they produced. The fact that the other biochemical tests, the serological reactions and the oxidation-reduction studies, support this division appears to us to be sufficient ground for proposing that groups I and II be designated as separate species of the genus, *Fusobacterium*.

The division of group I into two types by the fermentation of sucrose is not confirmed by the agglutination tests. Instead, the group appears to possess a complex antigenic structure. Although no evidence could be obtained that these serological subgroups are significant, a receptor-analysis study would in all probability yield more definite information.

The separation of group II into two types upon the basis of lactose fermentation was confirmed to some extent by the agglutination reactions. If the agglutinin-absorption results have any value, it is in the suggestion that types II A and II B are probably distinct entities. On the other hand, the precipitin tests failed to distinguish these two types.

Morphological and cultural characteristics may also serve as differential criteria for the groups, but since their taxonomic value is somewhat limited in the system employed here, they will be discussed separately in the second report of this series.

A comparison of the proposed classification with that of Slanetz and Rettger may be summarized as follows: Group I, type A, comprises Slanetz and Rettger's types I and II; group I, type B, contains a portion of their type III; group II, type A, contains the remainder of their type III; group II, type B, corresponds to their type IV.

#### CONCLUSIONS

1. Eighty strains of *Fusobacterium* were divided into two main groups by their biochemical and serological behavior.

<sup>2</sup> We are indebted to Dr. Gillespie for permission to make use here of these unpublished data.

2. Group II is distinguished from group I by (a) its ability to produce approximately twice as great an acidity from carbohydrates as the latter, (b) its property of fermenting maltose and trehalose, and (c) its inability to form indol, produce hydrogen sulfide and reduce nitrates.

3. Each of the two groups is made up of two important fermentative sub-groups, or types.

4. A method for preparing satisfactory agglutination antigens is described.

5. The precipitin technique failed to yield reliable results.

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## THE FUSOBACTERIUM GENUS

### II. SOME OBSERVATIONS ON GROWTH REQUIREMENTS AND VARIATION

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In the preceding paper of this series the writers separated the *Fusobacterium* genus into two main groups, on the basis of biochemical and serological properties. The present report deals with morphological and cultural characteristics of these groups, with emphasis on nutritional requirements and variation.

#### CELL MORPHOLOGY

The typical *Fusobacterium* cell is a Gram-negative, non-motile and non-sporulating bacillus which may or may not possess the classical fusiform shape. Following primary isolation, the cells become progressively smaller during the first ten or fifteen generations. Those of group I are shorter (averaging 3 micra) than those of group II (averaging 10 micra), type I A being the smallest, and type II B the largest. The group I bacilli are characterized also by their thinness (0.2 to 0.4 micron), by their pointed ends, and by their frequent production of granules. On the other hand, the cells of group II are thicker (averaging 0.6 micron), may have rounded ends, and contain chromatin material which is often distributed in a bizarre manner, even assuming at times a definitely bacillary or spirillar form. Group I shows a single contour in dark-field preparations, while group II is double-refractive. The characteristic morphologies are illustrated in figures 1 and 2.

<sup>1</sup> This paper covers part of a dissertation submitted by the Senior author to the Graduate School of Yale University in partial fulfilment of requirements for the degree of Doctor of Philosophy.

## CULTURAL CHARACTERISTICS

*Colony appearance.* The colony appearance of the various types progresses from the amorphous, convex and circular colony of type IA to the granular, flat and rhizoid colony of type II B. The manner of this transition resembles the smooth to rough change in other genera (figs. 3 and 4). Group II colonies may be detected readily with the naked eye by their ground-glass appearance when viewed with direct light. Isolated colonies are butyrous, grey and translucent. Those of group I, on the other hand, are more opaque, often show a brownish central area and are inclined to adhere to the medium.

*Potato extract medium.* Group I strains produce in potato-extract cysteine broth a dense, even turbidity, accompanied by a slight sedimentation after twenty-four hours' incubation, while group II cultures develop as a loosely floccular sediment, leaving the supernatant fluid practically clear. During the present investigation there were no exceptions to this differential behavior whenever vigorous growth occurred.

Varney (1927) stated that a foul odor accompanying his cultures invariably indicated the presence of a contaminating organism. Tunnicliff (1911), on the other hand, in agreement with most investigators, found her cultures to be offensive; Smith (1932) described one of his types as possessing a rancid odor. In the writers' experience, the nitrogen-active group I cultures, especially in the presence of cysteine, produced a distinctly pungent odor, predominantly that of  $H_2S$ . Group II strains, on the other hand, were faintly rancid, indicating the presence of butyric acid. The property of producing a disagreeable odor would seem to depend, then, upon the group to which a given organism belongs, as well as upon the medium employed.

*Oxygen relation.* One gains from the literature the impression that the fusiform bacilli differ widely in their tolerance of oxygen. Although they are usually reported as being strict anaerobes, Tunnicliff (1911), Larson and Barron (1915) and Sanarelli (1927) claim to have cultivated strains aerobically. It would appear, therefore, that either the fusobacteria acquire a tolerance for

oxygen, or that there is one type within the genus which is inherently anaerobic and another which is aerobic.

Recently-isolated cultures of *Fusobacterium* will grow only under strictly anaerobic conditions, and are extremely susceptible to atmospheric oxygen. We have observed that agar cultures which were allowed to remain exposed to the air for as short a period as one hour occasionally refused to develop on sub-culture. On the other hand, certain of the older strains survived long exposures. Fifty cultures isolated from three months to two years previously were tested for their ability to grow in open broth tubes, both with and without cysteine. Ten serial sub-cultures were made at forty-eight-hour intervals. At the end of the experiment twenty-three of the twenty-seven group I strains, four of the five intermediate and one of the eighteen group II cultures showed growth in the tubes containing 0.1 per cent cysteine. In the absence of cysteine, five of the group I, and none of the intermediate or group II strains developed. No growth was ever observed on the surface of agar plates incubated aerobically. The results of this experiment indicate the degree of oxygen tolerance that may be acquired, particularly by old group I cultures, under artificial cultivation.

Although old laboratory strains were less oxygen-sensitive than recent isolations, the necessity for using only freshly-prepared media cannot be over-emphasized. Neither broth nor agar, when allowed to absorb oxygen for a day or two, yielded satisfactory growth, even though the medium was boiled immediately before being used. The decrease in the growth-promoting qualities of aerated media may be related in part to the potato extract, since it quickly loses much of its growth-promoting property unless it is stored anaerobically.

#### NUTRITIVE REQUIREMENTS

Ordinary meat extract or meat infusion media will not support the growth of the fusobacteria. Although the addition of an aqueous potato extract was decidedly beneficial, many of our strains still developed poorly, or not at all. Fortunately this difficulty was partially overcome when it was found that the addi-

tion of small amounts of cysteine resulted in the abundant growth of all of the group I cultures. Since 0.1 per cent cysteine HCl serves to poise the medium at an OR level of approximately Eh-0.20, it is possible that the growth-promoting ability of this substance is due to its reducing property. On the other hand, the large quantities of  $H_2S$  produced in the presence of cysteine indicates that group I easily utilizes the sulphydril compound as a source of energy.

During the search for a similar substance for group II, it was observed that the addition of ordinary brown sugar, as reported by Allison and Hoover (1934) for *Rhizobium*, resulted in abundant growth. It was soon discovered, however, that any carbohydrate fermented by the given strains also possessed this property. The importance of carbohydrates as available sources of energy became apparent during the fermentation studies, when it was observed that most of the group II strains failed to grow at all unless a fermentable substance was present. As a result, glucose was added routinely to the basic medium.

After six months' cultivation in the laboratory, the majority of the group I and several of the group II cultures were able to develop without the potato extract. It is not unlikely, therefore, that some of the strains became trained, by artificial cultivation, to synthesize the substance essential for their development. A similar situation has been shown by Fildes and his co-workers (1933) to exist regarding the tryptophane requirements of *Eberthella typhosa*.

In table 1 are presented the results of an experiment designed to determine the relative value of glucose, cysteine and potato extract in promoting the growth of established laboratory cultures. Five strains of each type were cultivated in five different media, all containing as a base 1.0 per cent proteose peptone and 0.3 per cent Liebig's meat extract. The inoculum in every instance consisted of 0.1 cc. of culture, and transfers were made at twenty-four-hour intervals. Tubes showing no turbidity were re-incubated for an additional two days before being discarded. The experiment was terminated after the fifteenth sub-culture.

The substitution in the medium of neo-peptone for proteose peptone gave quite unsatisfactory results. The ineffectiveness of this ingredient is, perhaps, explained by the observation that the neo-peptone broth had a higher Eh value than the proteose peptone broth.

TABLE 1  
*Relative values of media constituents*

STRAIN	TYPE	LAST SUCCESSFUL SUBCULTURE IN BASIC BROTH CONTAINING:				
		No added substances	10 per cent potato extract	0.05 per cent cysteine HCl	0.1 per cent glucose	10 per cent potato extract; 0.05 per cent cysteine; 0.1 per cent glucose
VA <sub>8</sub>	I A	1	+	+	+	+
LA <sub>4</sub>	I A	1	+	+	10	+
V <sub>1</sub>	I A	1	+	13	5	+
Ch <sub>1</sub>	I A	1	+	+	+	+
R <sub>1</sub>	I A	4	+	+	+	+
H <sub>10</sub>	I B	1	+	+	+	+
VA <sub>1</sub>	I B	4	+	+	+	+
LA <sub>5</sub>	I B	1	3	+	5	+
M <sub>1</sub>	I B	0	+	+	+	+
D <sub>1</sub>	I B	5	+	+	+	+
H <sub>4</sub>	II A	0	+	1	2	+
H <sub>7</sub>	II A	0	1	0	1	+
H <sub>16</sub>	II A	6	+	+	+	+
H <sub>24</sub>	II A	0	+	0	+	+
VA <sub>3</sub>	II A	0	1	0	5	+
H <sub>27</sub>	II B	0	+	0	+	+
VA <sub>9</sub>	II B	0	1	0	+	+
LA <sub>7</sub>	II B	0	+	0	+	+
LA <sub>16</sub>	II B	0	+	+	+	+
GP <sub>1</sub>	II B	0	10	0	13	+

+ indicates growth at the end of the experiment; 1, 2, 3, etc. indicates last successful subculture; 0 indicates no growth.

#### VARIATION STUDIES

*Diffuse and granular growth in broth.* Under favorable conditions a vigorous group I culture develops as a diffuse suspension in broth. Group II, on the other hand, produces a loosely flocculent sediment. When favorable growth conditions do not



exist, however, the broth tubes of both groups may remain clear; however, upon agitation the sedimented material appears as granular particles in the case of group I strains, and as flocculent masses when group II is concerned.

The granular form of *Fusobacterium* appears whenever the activity of the strain has been permitted to lapse, or when the medium is not suitable for optimal development. Such cultures are unsuited for biochemical and serological studies. A series of rapid subcultures under favorable conditions, however, will, as a rule, restore the diffuse growth character. Inocula containing the granular cells ordinarily fail to develop on the special routine agar, but when growth does take place, the colonies are of a characteristic smooth nature. No morphological difference could be detected between the cells of the granular and the diffuse cultures of the same strains.

*Opaque variant.* Evidence of well-defined variation was first obtained when two distinct types of colonies (group I, type B) appeared spontaneously in a young agar plate culture. Whereas one, the smooth colony, was translucent and possessed a bluish-grey tinge, the variant type was opaque and dirty-white in color. Although of granular appearance, the latter type of colony was actually mucoid (fig. 5). A pure culture of the variant was easily secured. In broth it gave rise to an extremely filamentous, and at times membranous, growth (fig. 6). Biochemically, it was indistinguishable from the parent strain; agglutination tests could not be carried out, due to the mucoid nature of the growth. The precipitin tests were negative.

This sub-strain was relatively stable. Reversion to the smooth type, however, could be accomplished regularly by making several successive daily transfers. On the other hand, the translucent to opaque transition continued to occur spontaneously, unless frequent subcultures in meat infusion broth were carried out. Group II cultures produced the variant less frequently than group I. A study of the factors responsible for the appearance of the opaque colony indicated the importance of aging in broth and of a lowered vitality of the culture.

*Stability of the types.* The normal group II colonies possess

the rhizoid character one might expect in rough group I variants. Therefore, the stability of the types was of considerable interest. Although the transformation of one type to another had not occurred spontaneously, it seemed possible that drastic procedures might force the cells of one type to assume some of the characteristics of another. Consequently, two strains of each type were grown for ten generations in broth containing a 10 per cent concentration of specific antiserum. No significant morphological or biochemical change was observed; because of the granular nature of the growth, agglutination tests were not attempted.

#### RELATIONSHIP TO SPIROCHETES

The association of fusiform bacilli with spirochetes has attracted considerable attention in relation to the so-called fusospirochetal diseases. This relationship is generally regarded as being symbiotic, but Tunnicliff (1906) suggested that the spirillar form is, in fact, a stage in the evolution of the fusiform bacillus, the spirals appearing on the fourth or fifth day of incubation. These observations have received confirmation from Smith (1932), Sanarelli (1927) and others. However, most observers have found little resemblance between the spiral forms and true spirochetes. Tunnicliff (1923) further described the formation of these spirals within the large bacilli and their escape from the parent cell, following lysis. More recently (1932), the same author suggested that the spiral organisms are rough dissociants, since they were found by her only in the rough colonies; the smooth colonies contained bacillary cells exclusively.

An attempt was made by us to produce the spiral forms in a consistent manner, with the hope of fully determining the underlying factors. Nevertheless, the use of malt-extract blood agar, as employed by Tunnicliff, failed to produce more than an occasional spirillar element. In fact, growth upon this medium was meagre, due, perhaps, to the distinctly acid reaction (pH 6.2). In aging cultures small shrivelled cells were observed, but no evidence could be obtained that these forms were viable; they were not present in young sub-cultures, were refractive to vital stains such as neutral red, malachite green and crystal

violet, and could not be identified in dark-field preparations. Indeed, it became apparent that any medium having a distinctly acid reaction would stimulate the early production of involution and shadow forms, and an occasional spiral. The results of a number of experiments indicated that an acid medium and overheating of the film during the fixing process of staining were important factors in producing spiral cells. There was no observable difference in the ability of groups I and II to give rise to spirillar elements. It should be emphasized, however, that the occasions on which these spiral elements appeared were extremely few.

A second type of spiral was seen in young cultures as a long loosely-waved filament. These spiral forms were similar to those described by Varney in his type III strains. In spite of considerable effort spent in attempting to produce this type, it was rarely observed.

Eight strains, two from each type, were next subjected to a variety of procedures commonly employed to induce variation. Different media containing 0.5 per cent LiCl, 10 per cent specific antisera, and low concentrations of nutrient material failed to show evidence of spiral elements after fifteen sub-cultures, although a high degree of pleomorphism was noted. Growth in filtrates of *Bacillus mesentericus-vulgatus*, as suggested by Sanarelli, occurred only with group I strains; no spirals were observed in smears from these cultures.

Finally, upon the basis of Pilot's (1926) and of Sanarelli's observations that fusiform bacilli are generally found near the surface of morbid processes, whereas the spirochetel forms are most abundant in the depths of the lesions, the eight strains were inoculated into broth containing small pieces of fresh, sterile guinea pig liver. Repeated microscopic examinations of the growths and of impression smears made by gently pressing the surfaces of the tissues against slides revealed only bacillary forms.

These experiments may be summarized by stating that the spiral form does not appear to be a common morphological variant; on not more than a dozen occasions were these loosely waved filaments observed. Furthermore, no evidence was obtained

that the spirillar variant is characteristic of a single type, as suggested by Varney. None of the spirillar filaments could be mistaken for true spirochetes.

#### PATHOGENICITY STUDIES

The rôle of the fusobacteria in the so-called fuso-spirochetal diseases is still an open question. By most investigators the fusiform bacilli have been considered as secondary invaders, by some as saprophytes, and by a few as the primary etiological agents of many fetid and gangrenous processes. Their presence in smears from a wide variety of ulcerative and gangrenous conditions is complicated by the fact that these organisms are ordinarily found upon the normal mucous membranes of the mouth, throat and external genitalia. For a review of the literature on the pathogenicity of the genus, the reader is referred to the senior author's dissertation deposited in the Yale University Library.

In spite of the pathogenic rôle often attributed to these organisms, most investigators have failed to produce experimental infections with pure cultures (Lichtenburg *et al.*, 1933). Nevertheless, Tunnicliff and Klein (1934) produced subcutaneous abscesses in experimental animals with the aid of a glucoside. Without the aid of a chemical, Grumbach and Verdan (1936) reported the production of a fatal purulent peritonitis in guinea pigs. E. C. Smith (1933) noted in human volunteers a rise in temperature following the injection of pure cultures, which he interpreted as a manifestation of a toxic action. Two attempts (Pratt, 1927, and Cohen, 1933) to demonstrate the presence of a *Fusobacterium* toxin have yielded negative results.

During the preparation of antisera it was found that the intravenous injection of 1.0 cc. of strains VA<sub>1</sub>, LA, and GP<sub>1</sub> produced rapid death of rabbits. Positive cultures were obtained from the heart blood, lung and liver. A few preliminary virulence and toxicity experiments were performed, using white mice and rabbits; but, since there was insufficient opportunity to make a thorough study, the detailed results are omitted. In large doses these strains were lethal for mice when injected intrapleurally.

Less definite results were obtained by the intravenous route, and no abscess formation resulted from subcutaneous inoculations. The pathological picture at autopsy was indefinite and variable. Two months later the strains had entirely lost their virulence. Dick and Emge (1914) and Pratt (1927) have reported a similar loss of virulence by their strains.

Repeated efforts to demonstrate a soluble exotoxin, and an endotoxin prepared by alternate freezing and thawing, were negative. Likewise, the filtrates of young cultures failed to elicit the Shwartzman reaction.

#### DISCUSSION

In view of the fact that the fusiform bacilli readily fall into two groups of organisms which have widely different properties, it is conceivable why certain conflicting reports have appeared in the literature. The production of a disagreeable odor, for instance, is not a property of all strains of fusobacteria, but is limited to group I, especially when cysteine is present in the medium. Similarly, the fact that the strains studied by one investigator were reported as weakly saccharolytic, and as producing hydrogen sulfide, while those of another failed to form these substances, and at the same time were highly fermentative, does not necessarily exclude either one of the groups from this genus. Furthermore, contradictory reports concerning the necessity for an accessory group substance and for strict anaerobiosis may be explained by the observation that old laboratory strains are much less fastidious in their requirements than recently-isolated ones.

The usual criteria for determining roughness in bacterial colonies do not apply here. The typical group II colony possesses the granular composition, convoluted surface and rhizoid margin which generally characterize rough colonies.

Efforts to bring about the consistent formation of spirillar cells, the so-called spirochetal form, met with failure. While such a morphological type may exist, final proof of its occurrence in cultures possessing the biochemical and serological properties reviewed here still awaits demonstration.

## CONCLUSIONS

1. The morphological characteristics of the fusobacteria are variable, but conform in general to the proposed groups I and II.
2. The character of the growth in broth constitutes a valuable criterion for differentiating the two groups.
3. The growth requirements are of a widely divergent nature. A potato-extract medium containing glucose and cysteine supports the vigorous development of both groups.
4. The nutritive and anaerobic requirements are influenced greatly by artificial cultivation.
5. A granular type of growth in broth and an opaque colony on agar are manifestations of bacterial variation.
6. Spiral forms are, at best, of rare occurrence in laboratory media.
7. Some of the fusiform bacilli may be pathogenic for laboratory animals, but no evidence of toxin production could be obtained.

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## PLATE 1

FIG. 1. Cell morphology of strain Ch<sub>1</sub> (group I, type A). Forty-eight hour culture. × 1200 diameters.

FIG. 2. Cell morphology of strain LA<sub>9</sub> (group II, type B). Forty-eight hour culture. × 1200 diameters.

FIG. 3. Surface colonies of strain Ch<sub>1</sub> (group I, type A). Forty-eight hour culture. × 30 diameters.

FIG. 4. Surface colonies of strain LA<sub>9</sub> (group II, type B). Forty-eight hour culture. × 30 diameters.



FIG. 1



FIG. 2



FIG. 3



FIG. 4

(Earle H. Spaulding and Leo F. Rettger: *Fusobacterium* Genus)



## PLATE 2

FIG. 5 Variant and translucent opaque colonies of strain LA<sub>1</sub> (group I, type B) Forty-eight hour culture  $\times 30$  diameters

FIG. 6 Cell morphology of opaque variant from strain LA<sub>1</sub> (group I, type B) Forty-eight hour broth culture  $\times 1200$  diameters

FIG. 7 Cell morphology of translucent (smooth) colony of strain LA<sub>1</sub> Forty-eight hour culture  $\times 1200$  diameters ("Normal" colony)



FIG. 5



FIG. 6



FIG. 7

FIG. 5. Spaulding and FIG. 6. Reticker (Fusobacterium Genus)



# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## MICHIGAN BRANCH

JANUARY 15, 1937

A STATISTICAL ANALYSIS OF STANDARD PLATED COUNTS AND METHYLENE-BLUE PLATED COUNTS AND METHYLENE-BLUE REDUCTION TESTS MADE ON RAW MILK. *E. D. Devereux*, Department of Bacteriology, Michigan State College.

THE UNRELIABILITY OF EOSIN-METHYLENE-BLUE-AGAR IN THE ISOLATION OF COLI-AEROGENES GROUP. *Catherine Michael and W. L. Mallmann*, Department of Bacteriology, Michigan State College.

A COMPARATIVE STUDY OF METHODS FOR THE ISOLATION OF TYPHOID BACILLI FROM THE STOOLS OF SUSPECTED CARRIERS. *Elizabeth Cope and Joseph Kasper*, Department of Health, Detroit, Mich.

In order to determine the best method for use in the detection of carriers of *Eberthella typhosa*, a comparison of the results obtained by the use

of Endo and of bismuth sulphite agar was made. Endo plates were streaked from a suspension of approximately 1 gram of feces in 10 cc. of physiological salt solution. Bismuth sulphite agar plates were streaked from this suspension and directly from the stool specimen. Poured plates were also made using 5 cc. of the suspension and approximately 20 cc. of bismuth sulphite agar.

The specimens examined were obtained from two groups; food handlers who were suspected of being typhoid carriers, and persons who had been in contact with known cases of typhoid fever.

The results of this study were strikingly in favor of the use of poured plates of bismuth sulphite agar. Positive findings increased from 1.2 to 16.8 per cent among the food handler group, and from 8.4 to 17.5 per cent among the contacts when this method was employed.

## MICHIGAN BRANCH

FEBRUARY 12, 1937

INFLUENCE OF DEAD BACTERIA ON MICROSCOPIC COUNTS OF PASTEURIZED MILK. *Archibald R. Ward and Charles E. Myers*, Dairy Testing Laboratory, Detroit, Mich.

The experimental work reported upon, concerns the justification for the

widespread belief that bacteria killed by pasteurization, remain visible and are counted when microscopic counts are made of pasteurized milk.

The problem was studied by making microscopic counts of raw milk and of the same milk at short intervals during

and after the usual half-hour of holding at pasteurizing temperature. Percentages of reduction of bacterial count were computed from the count of the raw milk and from the counts obtained after pasteurizing for various periods.

Heating milk at pasteurizing temperature for 30 minutes caused a considerable reduction in the number of bacteria found in smears stained with methylene blue. The percentages of reduction obtained were comparable with similar figures that have been reported from studies made with plate counts. Most of the bacteria found after the 30-minute holding, disappeared after longer heating. Microscopic counts made at 5-minute intervals during pasteurization, revealed a generally consistent reduction in numbers of bacteria. The bacteria found in a well-stained condition were regarded as live bacteria which had survived the period of heating involved in each case.

The results support the belief that non-thermophilic bacteria in stained smears of pasteurized milk could hardly have given origin to the belief that dead bacteria interfere with microscopic counts. On the other hand, it is believed that before thermophilic bacteria were recognized as such they were mistaken for dead bacteria.

It was concluded that insufficient numbers of dead bacteria remain visible after pasteurization to impair the usefulness of direct microscopic counts made of pasteurized milk.

**RESULTS OF FIVE-YEAR QUALITY CONTROL PROGRAM ON DETROIT'S MILK SUPPLY.** *Russell R. Palmer*, Department of Health, Detroit, Mich.

Preliminary investigation of the causes of poor-keeping-quality milk as shipped from the individual farms, revealed that improper cooling, dirty

utensils, including dirty milking machines, badly infected udders, were the troublesome factors. Improper cooling accounted for over eighty per cent of the trouble with poor-keeping-quality milk.

Another preliminary survey revealed, that there was no relationship between the amount of milk shipped by a producer and the troubles experienced with unsatisfactory methylene blue or sediment tests.

The plan of procedure adopted after the preliminary work, was to exclude the shipper of unsatisfactory milk, as shown by methylene blue or sediment test, for varying periods of times, dependent upon the frequency of shipment of such unsatisfactory milk.

A review of the five years work in quality control in which the methylene blue test was used for the estimation of keeping quality of the milk and the sediment test used to determine the cleanliness revealed a steady and continual reduction in the milk judged to be unsatisfactory by the methylene blue test and a parallel improvement in the cleanliness of the supply.

Further study clearly demonstrated that the exclusion of the shipper of unsatisfactory milk, as judged by either test corrected the conditions, as demonstrated by the marked reduction in the number of producers that it was necessary to exclude more than a single time during the five-year period.

The conclusions are, that the use of tests to determine the fitness of milk for the market and the infliction of a penalty on the shippers of unsatisfactory milk will gradually, but surely improve the supply of incoming milk. It is evident that the shipment of unsatisfactory milk is due to carelessness on the part of the producer, and he must be penalized in some manner.

# THE GROWTH AND RESPIRATION OF SOME SOIL BACTERIA IN JUICES OF LEGUMINOUS AND NON-LEGUMINOUS PLANTS<sup>1</sup>

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Although many theories have been proposed to account for the specificity of the relationships between leguminous plants and root-nodule bacteria, there is little evidence to support any of them. Neither is it known why this relationship is confined to leguminous plants. Most of the studies have been made on the nodule bacteria apart from the host plants and hence natural conditions have seldom been approached.

Several investigators have shown that some plants develop an active immunity toward certain organisms. This immunity is generally localized near the point of injection or of the previous infection. Chester (1933) has given a review of the subject and only a few references will be mentioned here.

Hiltner (1902) and Hiltner and Störmer (1903) found that the expressed sap of leguminous plants caused plasmolysis and a breakdown of the nodule bacteria. They concluded that the reactions of host and bacteria are best explained in terms of a partial or controlled active immunity. Süchting (1904) recognized the acquired immunological character of this relationship, in part, as a result of his observation that there is a limit to the number of nodules, regardless of the amount of inoculum introduced.

Cappelletti (1924, 1926) noted a relationship between the production of antibodies by the legume plant and the formation of bacteroids, suggesting that the *Leguminosae* comprise two dis-

<sup>1</sup> Journal paper J-458, Iowa Agricultural Experiment Station, Ames, Iowa Project No. 226.

tinct types. In one, typified by *Pisum*, bacteroids are formed accompanied by nuclear disturbances and the production of antibodies specifically directed against the bacteria. In the other, typified by *Phaseolus*, no bacteroids or antibodies occur in the nodules. He observed that bacteroids made their appearance about the time the plant flowered. Agglutinins and precipitins were also present only at this time, being absent in the younger nodules which contained no bacteroids and disappearing at the stage of seed production. The antibodies were strictly localized in the root-nodules, and were destroyed by heating at 78 to 80° for one-half hour. While Cappelletti's work helps to explain some of the bacterial host-plant relationships, it fails to indicate anything concerning the initiation of these relationships.

Several investigators have employed various extracts of leguminous plants for the culture of *Rhizobium* (Allison, 1927 and Itano and Matsuura, 1936). No investigations have come to our attention, however, in which growth has been studied directly in the plant sap. Sherman and Hodge (1936) demonstrated that juices expressed from cabbage heads and the roots of turnips possessed mild but distinct bactericidal properties, but this was not the case with the juices from carrots, parsnips and cucumbers. The bactericidal property of the cabbage and turnip juices was destroyed at 60°C. for 10 minutes, adsorbed by activated carbon and removed by a Berkefeld N filter, but not by the V candle. Such a bactericidal property of plant juices is of interest in the study of the relation between legume plants and the nodule bacteria.

In view of all information available regarding the activity of the root-nodule bacteria in artificial media, it seems that studies made directly in the freshly expressed juices of plants may be very helpful in correlating our present knowledge with the processes involved in symbiosis.

#### PROCEDURE

The fresh plant material was minced and pressed in a Carver laboratory press at about 4000 lbs. per square inch. The juice

was clarified by centrifuging in a Sharpless super-centrifuge at 35,000 revolutions per minute for 10 minutes and passing through fine filter paper. Sterilization was accomplished by filtration through L<sub>3</sub> Chamberland-Pasteur filters. Sterile filtrates were not obtained with number N Berkefeld filters.

In each experiment a portion of the clarified juice was heated in streaming steam for 5 minutes, which precipitated the proteins, consisting mostly of albumins. The remainder of the juice was kept cold from the time it was expressed until it was sterilized and inoculated. For this purpose the containers were packed in ice as much of the time as possible. It was hoped by these precautions to keep any changes due to enzymatic action at a minimum.

Ten-cubic centimeter portions of the sterile juice were then transferred aseptically to test tubes and inoculated. The usual procedure required about 3 hours from the time the plants were harvested until the juice was inoculated. Inoculations were made with 7 cc. of 24 to 48-hour cultures, which had been previously diluted to the desired concentration with sterile physiological salt solution. The cultures were grown in a mineral salts solution of the composition employed in previous investigations (Thorne and Walker, 1936). Commercial cane sugar was added as a source of energy and either asparagin or KNO<sub>3</sub> as the nitrogen source.

The cultures were incubated at 28°C. and shaken at various intervals. In most instances numbers of organisms were determined by direct counts with a Petroff-Hausser chamber. In a few instances counts were made by the agar plate method.

The following strains of bacteria were used: *Rhizobium meliloti* 110 and 132, *Rhizobium trifolii* 206, *Rhizobium leguminosarum* 301, *Rhizobium japonicum* 403, *Rhizobium phaseoli* 504, cowpea bacteria 606, *Achromobacter radiobacter* 1 and 2, *Pseudomonas pyocyanea*, and *Bacillus subtilis*.

The pH of the various juices was determined with either quinhydrone or glass electrodes. Nitrogen determinations were made by the Kjeldahl method.



## RESULTS

*Growth studies*

In preliminary experiments it was observed that juices which had been heated often permitted the growth of bacteria which showed no activity in unheated juice. The growth of several species was therefore studied in heated and unheated portions of juices from different types of plants. The data of a representative determination are given in table 1. In each case duplicate cultures were made and the average reported.

The results of several experiments, including the data in table 1 are summarized in table 2. In this table, data representative of

TABLE 1

*Growth of bacteria in juice expressed from the tops and roots of navy bean plants*

ORGANISM	INOCU- LUM	ROOTS				TOPS			
		Unheated		Heated		Unheated		Heated	
		24	48	24	48	24	48	24	48
		hours	hours	hours	hours	hours	hours	hours	hours
millions per c.									
R. phaseoli.....	6 86	3 334	2 778	28 056	212 78	6 888	7 778	5 278	20 832
R. japonicum.....	4 16	3 334	28 054	25 834	142 25	4 166	45 832	1 944	2 222
R. meliloti.....	7 44	15 274	13 612	34 444	118 73	35 556	31 888	32 778	53 04
A. radiobacter.....	10 44	0 0	0 66	61 667	210 50	9 166	5 556	58 610	13 334
P. pyocyanea.....	9.86	138.27	400 +	144 25	400 +	123 3	400 +	130 05	400. +

those secured with the juice from each type of plant studied are reported.

Several tests were made with juice from different parts of the soybean plant and with different organisms. In general, the growth-inhibiting substances were most potent in the juice from the roots, nearly as strong in that from the leaves and somewhat weaker in that from the stems.

The soybean organisms grew slowly in both the heated and unheated juice. In this respect the soybean and the closely related cowpea organisms differed from most of the other bacteria studied. With the exception of *Pseudomonas pyocyanea*, the other species tested were strongly inhibited by the unheated juice. The bactericidal effect of the unheated juice was particularly

noticeable with *Achromobacter radiobacter*. Often a definite growth was noted with this organism for a few hours, followed by an agglutination or a precipitation of many of the cells. In many instances after 24 to 48 hours no cells could be seen with the microscope. Apparently agglutination was often followed by a breakdown of the cells. The destruction of the organisms was not complete, however, for after 5 or 6 days a good growth would appear in most of the tubes. Similar effects were noted with alfalfa and red clover bacteria, but to a somewhat lesser degree.

The results with juices from alfalfa and garden pea plants were similar to those obtained with the juice of soybeans. In both cases the nodule bacteria grew as well (or better) in the unheated as in the heated juice of the host plants. As before, it was found that organisms closely related to the nodule bacteria were benefited by the heating of the juice.

The results with navy beans differed from those of the previous experiments in that the unheated juice from the plant roots was bactericidal to *R. phaseoli*. No definite growth of this organism was observed in the unheated juice from the tops, but in one instance growth was observed in unheated juice from young plants. *A. radiobacter* made a good initial growth in heated juice from the tops, as shown in table 1, but was later partially agglutinated and a lower count was recorded after 48 hours. Other organisms reacted in a similar manner with juices of other leguminous plants.

The results with *P. pyocyanea* were different from those secured with other organisms. In no instance was inhibition of the growth of this species noted. A few tests with *Flavobacterium suaveolens* indicated that this organism is also able to make good growth in both heated and unheated juices. In most cases *Bacillus subtilis* did not seem well adapted to growth in these media. It was noted, however, that in general with this organism there was a much thicker pellicle formed on the heated juices than on the unheated. Furthermore, these juices did not seem conducive to the formation of chains, the cells usually occurring singly or in pairs.

The juices from 2 non-leguminous plants were also studied. In

TABLE 2

*The growth of bacteria in expressed juice of various plants in 36 to 48 hours*  
Inoculation = 3 to 10 million organisms per cubic centimeter

SOURCE OF JUICE		pH	RHIZORIUM JAPONICUM	CORYNE BACTERIUM	RHIZORIUM MELILOTI	RHIZORIUM TRIFOII	RHIZORIUM LEGUMINOSARUM	RHIZORIUM PASPALI	ACETOBACTER RADIOBACTER	PSEUDOMONAS PYOCYANEA	BACILLUS SUBTILIS	CONDITION OF PLANTS
Soybean	leaves	U* 6.5	+++						+++			Very few nodules 3 to 4 weeks of age
		H 6.4	+++						+++			
	stems	U 6.4	+++						+++			
Soybean	roots	U 6.6	+++						+++			Well nodulated 4 weeks of age
		H 6.6	+++						+++			
	complete	U 6.3	+++	+++	+++	+++			+++	+++	++	
Alfalfa	complete	H 6.3	+++	+++	+++	+++			+++	+++	++	Well nodulated 6 weeks of age
		U 6.9			++	++			++			
		H 6.9			++	++			++			
Peas	complete	U 6.3			+++	+++	+++	++	+++			Few nodules 3 weeks of age
		H 6.4			+++	+++	+++	++	+++			
		U 6.7	++		-			++	-	++	++	
Navy beans	tops	H 6.7	+		++			++	+	++	++	Well nodulated Blossoming age
		U 6.7	++		-			++	-	++	++	
	roots	H 6.7	+++		+++			++	+++	+++	+++	
Navy beans	complete	U 6.75	++		+++			++	++	+++	+++	3 weeks of age, few nodules
		H 6.75	-		+++			++	++	+++	+++	
					+++			++	++	+++	+++	

Corn complete.. .. .	{ U 5.57 H 5.55 }	-- +	-- -	-- -	-- -	-- -	2 to 3 weeks of age
Wheat complete.....	{ U 7.2 H 7.2 }	++ +	-- ++	-- ++	-- ++	-- ++	3 weeks of age
++++ = Growth of 400 million organisms per cubic centimeter or greater.							
+++ = Growth of 150 to 400 million organisms per cubic centimeter.							
++ = Definite positive growth.							
+ = Questionable growth.							
- = No growth							
-- = Decrease in number of organisms.							
--- = Organisms almost entirely disappeared.							
U* = Juice unheated.							
H = Juice heated.							
† = Organism which forms nodules on plant from which the juice was obtained.							

the case of corn the low acidity of the juice was at least an important factor in preventing any appreciable growth of the organisms. It was noted, however, that in the unheated juice there was a definite decrease in the number of organisms. In the heated juice the decrease was not appreciable, and in the case of *R. japonicum* a slight increase in numbers was noted.

In the juice from wheat there was a favorable reaction upon the growth of the organisms being studied. In this instance the soybean organism and *P. pyocyanea* grew better in the unheated juice than in the heated. The other bacteria encountered a definite toxicity in the unheated juice. *B. radiobacter* made no growth in either instance.

### *Respiration studies*

The oxygen utilization of some species of the nodule bacteria was studied in juices from certain leguminous plants. The study was conducted in Warburg manometers employing a technique similar to that of previous investigations (Thorne and Walker, 1936). One cubic centimeter of the sterile juices or medium was placed in each manometer vessel and diluted with 1 cc. of a suspension of organisms. The organisms were obtained from cultures which had grown about 48 hours in the medium given above. The cells were washed twice and suspended in a phosphate-buffered solution such that a concentration of about 10 billion organisms per cubic centimeter was usually obtained.

The results with the juices of alfalfa and soybean plants showed similar trends. The data from a representative experiment in which *R. japonicum* and *R. meliloti* were studied in juice from soybeans are shown in figure 1. The medium A employed as a comparison with the plant juices contained the mineral salts listed previously. To this was added 1 per cent of sucrose c.p. and 100 p.p.m. of nitrogen as  $\text{KNO}_3$ .

In figure 1 the logarithms of the oxygen consumed per one-half-hour intervals are shown plotted against time. The curves indicate that in all cases the rate of increase of oxygen utilization was approximately logarithmic for definite periods. In the case of *R. meliloti* this period was longer in the heated juice than in the

unheated, while with *R. japonicum* the logarithmic increase continued for a greater time in the unheated juice. In both instances the rate of oxygen consumption was greater in the heated juice

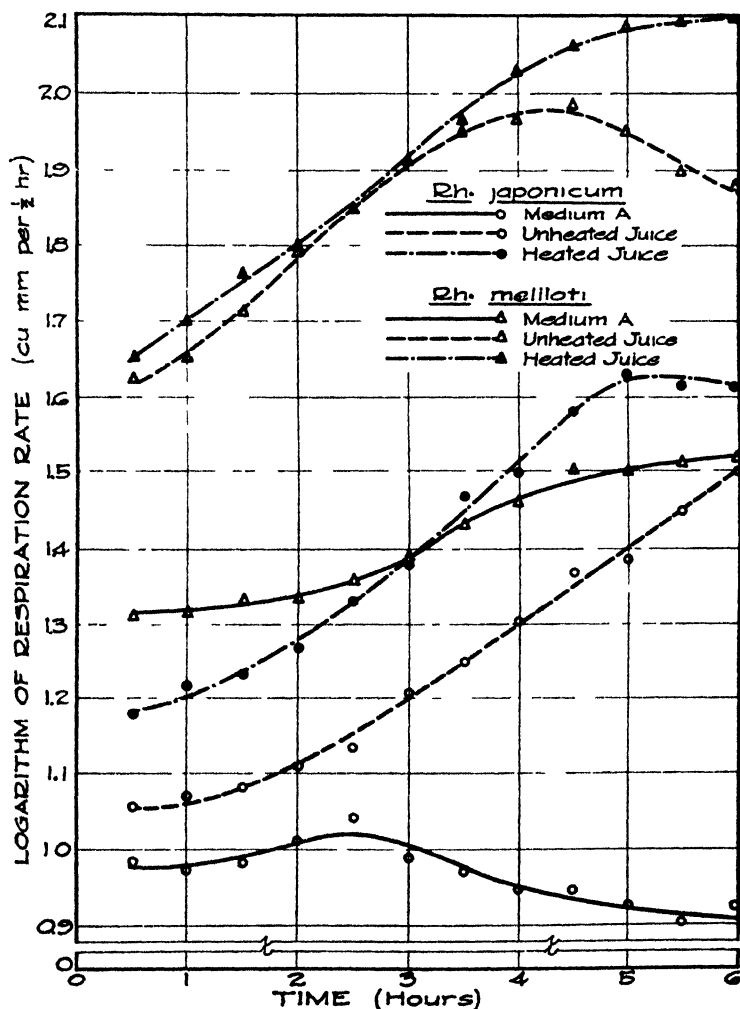


FIG. 1. THE RATE OF RESPIRATION OF *Rhizobium meliloti* AND *Rhizobium japonicum* IN JUICE EXPRESSED FROM SOYBEAN PLANTS

than in the unheated. The soybean organism, however, was benefited to a greater extent by the heating of the juice than was the alfalfa organism. These results are somewhat different from

those obtained in the study of the growth of these bacteria, although the data are not entirely comparable. The difference between the results of the growth and respiration studies may be due to the dilution of the juice, the much greater number of organisms in the inoculum, and the shorter duration of the experiments in the respiration studies.

There was a large increase in the rate of respiration in the juices studied over that in medium A. The rate of respiration of these organisms in soybean juice diluted with an equal volume of medium before placing in the manometer vessels was closely similar to that given in figure 1 for the juices diluted only with the suspension of organisms.

#### CHARACTERISTICS OF THE GROWTH-INHIBITING PRINCIPLE

In view of the decided bactericidal effect of the unheated juices of leguminous plants toward *A. radiobacter*, a further study was made to characterize some of the properties of the toxic principle. Juice from soybean plants of 3 to 4 weeks of age was employed. Treatments of the plants previous to expressing the juice, with the exception of heating, had no noticeable effect on the agglutinating or bactericidal properties. The treatments studied included freezing, grinding, mincing and heating in streaming steam for 5 minutes. Plants given the latter treatment yielded a juice affecting the organisms in a manner similar to that of the heated juice.

It was found that heating the juice for 10 minutes destroyed the bactericidal property at 65°C., and partly destroyed it at 60°C. The principle was only partially destroyed, however, at 53°C., which was the point at which the albumins present coagulated. The nitrogen content of the material precipitated during the heating amounted to about 28 per cent of the total.

The potency of the factor also decreased with time. After 10 hours at 25°C. some decrease in strength could be noted. After 24 hours only slight inhibition was observed. The factor was adsorbed by 10 grams of bone charcoal or norite from 75 cc. of juice, but was only partially removed by a moderate treatment with 2 grams of charcoal per 50 cc. of juice. Filtration through

N Berkefeld filter candles or L<sub>3</sub> Chamberland-Pasteur filters did not seem to remove any appreciable amount of the principle. It was precipitated by neutral lead acetate, but only partially removed by precipitation with barium acetate. Attempts to precipitate it with ammonium sulfate were inconclusive since the high concentrations of this salt remaining within the juice also agglutinated the organisms.

These results seem to indicate that the factor is closely associated with the protein fraction of the plant juices. It also seems to be closely related to the principle noted in the juice from cabbage and turnip plants by Sherman and Hodge (1936). The factor in the juices from leguminous plants, however, passed through filters which would not permit the passage of the principle studied by Sherman and Hodge. Furthermore, the agglutinating effect in the juices from legumes did not seem to be any greater in the clarified juice than in that passed through filter candles.

#### DISCUSSION

The following points seem to indicate that the growth-inhibiting substance in the juices studied was different from the antibodies noted by Cappelletti: (1) The principle was, with the exception of *Phaseolus*, not effective against the specific organism infecting the plant. (2) The principle was not specific for any one organism. (3) It was not localized in the root nodules. (4) The plants, in most cases, were used before the flowering stage when the antibodies are reported to make their first appearance. Furthermore, the nodules on the plants employed were in all cases the result of chance inoculation and usually were not numerous.

The growth-inhibiting principle studied is probably different from the factor encountered by Allison (1927) and Itano and Matsuura (1936) in extracts of leguminous and non-leguminous plants since in the present case it was destroyed by heat. In the legume extracts the growth-inhibiting property seemed to be a function of concentration and was not destroyed by heat.

The factor may be related to a principle found in extracts of legume seeds and plant materials. The studies of Wilenko (1910),



Raubitschek and Wilenko (1910), and Baldwin, Fred and Hastings (1927) indicate that the agglutinin and precipitin properties of such extracts are due to phytoalbumins which may form precipitates with certain bacterial and animal cells, or extracts of these cells. It does not seem probable that such a protein antagonism could account for all of the bactericidal effects noted since in some instances there was inhibition of growth or decrease in bacterial numbers when no agglutination or precipitation occurred.

Glaser and Prinz (1926) have shown that common enzyme systems, such as oxidases, may possess definite bactericidal properties. Since unheated plant juices are rich in such enzyme systems it is not improbable that they may play an important rôle in some of the effects noted. Little is known of the influence of such systems on the organisms studied and hence no conclusion is possible.

It seems that a study of expressed juices of legume plants may help to clarify the problem of legume-bacteria symbiosis. The data reported indicate that while root-nodule bacteria do not make rapid growth in juice freshly expressed from their host plants, they are much less adversely affected by it than are other closely related organisms. This seems true with bacteria as closely related as *R. meliloti* and *R. trifolii*. It is quite probable that this effect plays an important rôle in cross inoculation.

The results with *R. phaseoli* and the juice expressed from beans are somewhat at variance with the above conclusions. The work of Cappelletti (1926) showing that the bean plant does not react like most legumes towards the related organism is suggestive of a difference in host-plant bacterial relationships in this instance. Several other workers (Fred, Baldwin and McCoy, 1932) have also observed that this association seems different from that of other leguminous plants. This difference in relationship is in harmony with the results of these experiments.

#### SUMMARY

1. The growth and respiration of several species of bacteria have been studied in juices expressed from different types of plants in a study of the problem of legume symbiosis.

2. Most of the legume bacteria studied were able to grow in freshly expressed juices of their host plants, but such juices were bactericidal to other species of root-nodule bacteria and closely related organisms.

3. The cowpea bacteria grew as well as *Rhizobium japonicum* in the juice or soybeans.

4. The juice of navy bean plants was bactericidal to *Rhizobium phaseoli* in some instances. The relationships in this association are probably different from those in other cross inoculation groups.

5. The juices studied were in no instance bactericidal to *Pseudomonas pyocyanea*.

6. The bactericidal effects noted varied from inhibition of growth to agglutination and precipitation and probably a breakdown of the cells.

7. The bactericidal properties of the juices seem to be associated with the protein fraction.

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# AN APPARATUS FOR MEASURING TURBIDITY OF BACTERIAL SUSPENSIONS

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The authors have developed an inexpensive apparatus for measuring relative turbidities in convenient numerical units which requires no special skill for its operation. Its construction is illustrated in figure 1. It is essentially a black box 2.5 x 3.5 x 10 inches in size. In one end is mounted a Weston Photronic cell connected to a microammeter. In the other end of the box are four baffles extending from the sides toward the center and pressing firmly against a test tube which is admitted through a hole in the top. The edges of the baffles nearest the test tube have black velvet glued over them so as to make a light-tight seal against the glass. An intense beam of light from a projection lantern enters the box from one side to illuminate the test tube containing the suspension. Part of the light scattered at right angles to the beam from the lantern illuminates the Photronic cell and a definite reading appears on the microammeter. With this arrangement a tube of bubble-free distilled water will scatter almost no light so that the reading of the microammeter is practically zero, while, if the tube contains a cloudy suspension the reading will be higher.

The use of the apparatus is illustrated by the curves shown in figure 2. Curve A shows the way the microammeter reading for a broth culture of *Staphylococcus aureus* increased during incubation at 37°C. To obtain the readings the tube was removed from the incubator at intervals, shaken, inserted in the apparatus, and the microammeter reading taken. Curve B shows the results for a similar suspension to which an antiseptic was added at the time indicated by the arrow. Curve C also shows a similar

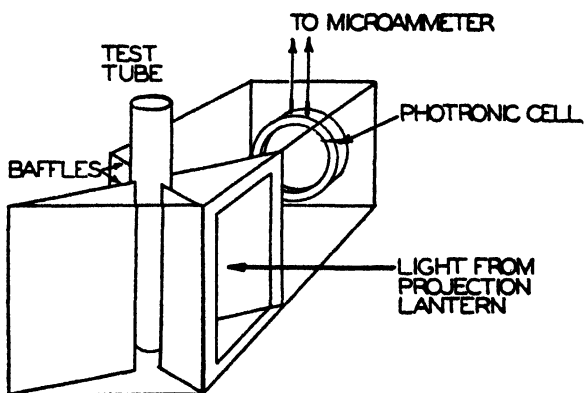
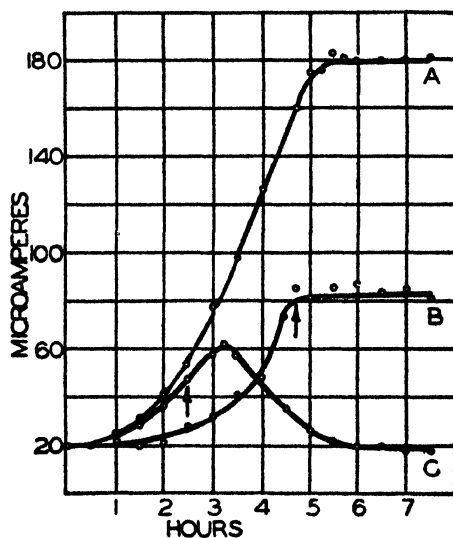


FIG. 1. DRAWING OF THE APPARATUS FOR MEASURING TURBIDITY

FIG. 2. READING OF THE MICROAMMETER FOR A BROTH CULTURE OF *STAPHYLOCOCCUS AUREUS* DURING INCUBATION AT 37°C.

Curve A, normal culture; curve B, culture to which an antiseptic had been added at the time indicated by the arrow; curve C, culture to which an homologous bacteriophage had been added at the time indicated by the arrow.

suspension to which an homologous bacteriophage was added at the time shown by the arrow. The curves clearly show that when the growth proceeds uninterrupted the readings are progres-

sively higher. When the growth is stopped by the antiseptic the turbidity immediately becomes constant, while after the addition of the bacteriophage the turbidity increases for an interval and then decreases during the clearing period to the original value.

One may use tubes containing standard suspensions of Fuller's earth to calibrate this apparatus and change the scale of the microammeter so that it will read the turbidity directly according to Fuller's scale.



# THE RELATION OF PEPTONE TO PRODUCTION OF HAEMOLYSIN BY STREPTOCOCCI<sup>1</sup>

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Any attempt at quantitative estimation of streptococcal haemolysin must take into account a number of difficulties attending such a determination. In the first place this haemolytic activity is not due to a single substance. Todd (1932, 1934) showed that under certain conditions even Group A streptococci produce an oxygen-stable non-antigenic haemolysin, as well as the oxygen-labile, antigenic lysin. Furthermore, while the haemolysin developed in serum broth is antigenic, the antiserum will neutralise only Todd's oxygen-labile lysin, and not the homologous antigen (Todd, 1933). It is not known whether this is due to the presence of two lytic factors when the streptococci are grown in the presence of serum. There are thus two, possibly three, haemolytic substances to consider.

One of these substances appears in broth filtrates wholly or partly in the oxidised state, and is only haemolytic after reduction (Neill and Mallory, 1926; Todd, 1932). Hodge and Swift (1933) demonstrated the importance of allowing sufficient time to elapse for complete reduction of the oxidised haemolysin before carrying out the titration.

Variable and unpredictable loss during the process of filtration adds a third difficulty to attempts to titrate the exact yield of haemolysin. The fact that cultures grown in the presence of serum yield a much more easily filterable lysin than do those grown without serum merely combines two of these factors which are difficult to control.

<sup>1</sup> This work was aided by a grant from the Banting Research Foundation, Toronto, Ont.



The foregoing considerations deal with that fraction of streptococcal haemolysin which escapes from the organisms into the culture fluid. The conditions which determine this escape are difficult to control and imperfectly understood. Todd himself commented on the irregularity with which different batches of his original yeast-extract broth would produce filterable lysin, although no reason could be assigned for the variability. The experience of the author with the medium described later by Todd and Hewitt (1932) has been that storage in the ice-box induces the formation of a crystalline deposit, after which it will not produce filterable haemolysin, even though the whole culture is actively haemolytic. The crystals appear to be chiefly composed of triple phosphate, as one might expect in a meat infusion reinforced with sodium phosphate and adjusted to pH 8.0. There is, however, an organic component which repeated washing of the crystals in water fails to remove. Elimination of excess phosphate in preparing this broth does not so completely prevent the formation of filterable lysin, as does this slow crystallisation. Failure of the haemolysin to dissociate from the streptococci may be due to loss of an essential organic substance in this crystalline complex. The existence of such "solvent" action on haemolysin was demonstrated in serum by Weld (1934, 1935).

It seems clear that estimation of the total amount of haemolysin formed must take into account both the filterable and non-filterable fractions.

As the experiments described in this and the succeeding paper progressed, it became increasingly clear that in such infusion broth the haemolysin remained intimately associated with the streptococci, even though the superficial nature of the property was attested by the prompt liberation of the haemolysin on shaking the washed organisms with serum for two minutes. No evidence was found of the escape of any haemolysin into the medium. With no differentiation in location of the haemolysin, and therefore in its availability for erythrocytes, it is much less probable that such variables as the ratio of filterable to non-filterable lysin, the species of erythrocyte selected, and the time of incubation of the test, will influence the final determination.

It was considered justifiable, therefore, to estimate the total amount of haemolysin formed, by titration of the whole culture. It is vital to the method to establish the fact that haemolysis by living culture diluted in saline and incubated with erythrocytes for one hour at 37°C. is not a manifestation of continued cell division or of metabolic activity *per se*. Actual colony counts were carried out on the erythrocyte-culture mixtures before and after incubation. Poured plate cultures in standard laboratory agar were made with dilutions of the mixture in saline. The plates were incubated for two days aerobically. The number of colonies after incubation of the erythrocyte mixture was never increased, and usually showed a definite reduction. The significance of this reduction will be made clear in a subsequent communication. Ordinarily bacterial haemolysin titrations are not carried out with strict aseptic technique. In a typical experiment where asepsis was observed to avoid errors in colony counts, five individual plate counts made from one reacting tube before and after incubation of the lysin titration were:

Before.....	56	58	61	54	46	Average 55
After.....	51	40	49	38	42	Average 44

The dilutions of culture were made with Kimble Exax pipettes. Successive shakings of the erythrocyte-culture mixture could only tend, by rupture of the streptococcal chains, to increase the colony count. Smears of the reacting mixture do not suggest that failure to demonstrate an increased colony count is due to agglutination or adsorption of the streptococci by the erythrocytes. In the reacting mixture from which the above counts were made the ratio viable streptococci/erythrocytes was approximately 100/1, and any such adsorption should have been easily visible.

It must be concluded, therefore, that haemolysis by living streptococci during a one-hour period at 37°C. is not due to continued multiplication of the cocci. Moreover, if dilutions of the same 16-hour culture are made in saline and in the broth devised for optimal lysin production, incubation with erythrocytes always shows a higher titre of haemolysin in the tubes

diluted with saline. This is partly referable to the fact that streptococci transplanted to a medium optimal for their multiplication do not show haemolytic activity for a period of at least two hours. It is probable, too, that the diluent influences the ease of transfer of the haemolysin from the bacterial cell to the erythrocytes.

Additional support for the contention that whole culture titrations afford a measure of the amount of haemolysin actually present in or on the bacterial cells at any given time is forthcoming from the observations of Mudd *et al.* (1937). These workers have produced evidence that the oxygen-labile haemolysin studied by Neill and Mallory, and Todd, is part of the labile surface antigen complex which contains in addition Lancefield's M and C substances.

The validity of whole-culture titration really turns on the ease and constancy with which formed streptolysin is transferred from the bacterial cells to the erythrocytes contacting them. If the theory is substantiated that liberation of the haemolysin from the streptococci into medium, serum, or erythrocyte suspension depends on the lipoids of these extracting agents, one of the chief difficulties in accepting whole-culture titration will disappear.

With these reservations in mind, in the experiments here recorded serial dilutions of whole culture in saline were tested for their capacity to lyse rabbit erythrocytes. No insistence is placed on the precise relationship of the haemolysin so titrated to the lytic principles reviewed above, although it seems probable that incubation at 37°C. ensures the elimination from consideration of Todd's oxygen-stable haemolysin.

It may reasonably be maintained that a proportion of the haemolysin still attached to the bacterial cell may be in the oxidised, inactive state, and so escape demonstration. It was never possible, however, to increase the lytic activity of a culture by reduction with sodium hydrosulphite. The protective action of serum against oxidation of the haemolysin raises the possibility that similar protection is afforded by the bacterial cell proteins.

## METHODS

The basic meat infusion was prepared from fresh beef heart, minced and infused in distilled water for two hours at a temperature of 75° to 80°C. Murray and Ayrton (1924) showed that extracts made between these temperatures were richer in coagulable proteins and contained accessory growth factors which were not liberated from the meat above or below these temperatures.

Other materials were added to the infusion in the required concentration. The coagulable proteins were removed by heating at pH 7.0 in a water bath. Determinations of pH were made colorimetrically. Frequently a second pH adjustment and heating were necessary to obtain a medium that was still clear after sterilisation. There was no marked or constant difference between centrifuging down these deposits and filtering them off through Whatman No. 2 paper. Prior to filtering the broth the filter paper was well washed with warm distilled water to remove the considerable amount of soluble salts contained in it, even though no deleterious effects were noted in the absence of such washing. Sterilisation was effected by autoclaving for twenty minutes at 120°C. Filtration through Seitz or Pasteur-Chamberland filters gave no markedly better results, even though the heat coagulation of the meat infusion proteins was thereby rendered unnecessary.

Lyall observed in 1914 that 2 per cent of peptone was more favorable than 1 per cent for haemolysin formation, and the early experiments in this series were suggestive of the extreme importance of peptone, both in obtaining maximum growth, and in the development of haemolysin. Neopeptone (Difco) proved to yield much larger growths and a more potent haemolysin than other commercial peptones or a tryptic digest of heart muscle prepared in the laboratory, and has been used throughout these experiments, except where otherwise stated. The other components of the finished broth were used on the basis of experiments described in a second paper.

Throughout the tests recorded in this paper the media were

inoculated with *Streptococcus pyogenes*, strain 206, isolated from a fatal case of septicaemia. Other strains tested routinely for haemolytic activity gave concordant results. Cultures were incubated for sixteen hours aerobically and to dilutions of whole culture in saline, in volumes of 1.0 cc., was added 0.5 cc. of a 5 per cent suspension of rabbit erythrocytes. Tests were incubated one hour in a water bath at 37°C. This incubation period did not permit lysis through multiplication of the organisms during the test. Degrees of haemolysis were recorded as: 4 (complete), 3 (75 per cent), 2 (50 per cent), 1 (25 per cent), tr (trace).

#### THE CONCENTRATION OF PEPTONE

Batches of broth containing the concentrations of Neopeptone shown in table 1 were adjusted to pH 7.0; 0.2 per cent NaCl,

TABLE 1

PEPTONE	CUBIC CENTIMETERS CULTURE											GROWTH OPACITY OF CULTURE
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	
per cent												
0	tr.	0	0	0	0	0	0	0	0	0	0	+
4	4	3	2	1	tr.	0	0	0	0	0	0	++++
6	4	4	4	3	2	tr.	0	0	0	0	0	+++++
8	4	4	4	4	4	3	2	1	tr.	0	0	+++++
10	4	4	4	4	4	4	3	2	1	tr.	tr.	+++
12	4	4	4	4	4	4	4	3	2	1	tr.	++
15	4	4	4	4	4	4	3	2	1	tr.	0	+
20	4	4	4	4	4	3	2	1	0	0	0	±

0.2 per cent  $\text{NaHCO}_3$ , 0.25 per cent glucose were added prior to sterilisation. Five cubic centimetre quantities were inoculated with strain 206. Table 1 gives the haemolysin titres at the end of sixteen hours growth at 37°C.

Contrary to the findings of Cook (1921), there is no correlation between the rate of multiplication of the organisms and their haemolytic activity. The lysin content of the culture in 20 per cent peptone broth is remarkable considering the paucity of growth. Needless to say, the inability of uninoculated medium to lyse erythrocytes was proved by controls.

This optimal concentration of peptone is a constant finding,

and the optimum varies with the batch of peptone. The quality of the individual lot of peptone varies very considerably. To represent graphically the relation of haemolysin to peptone

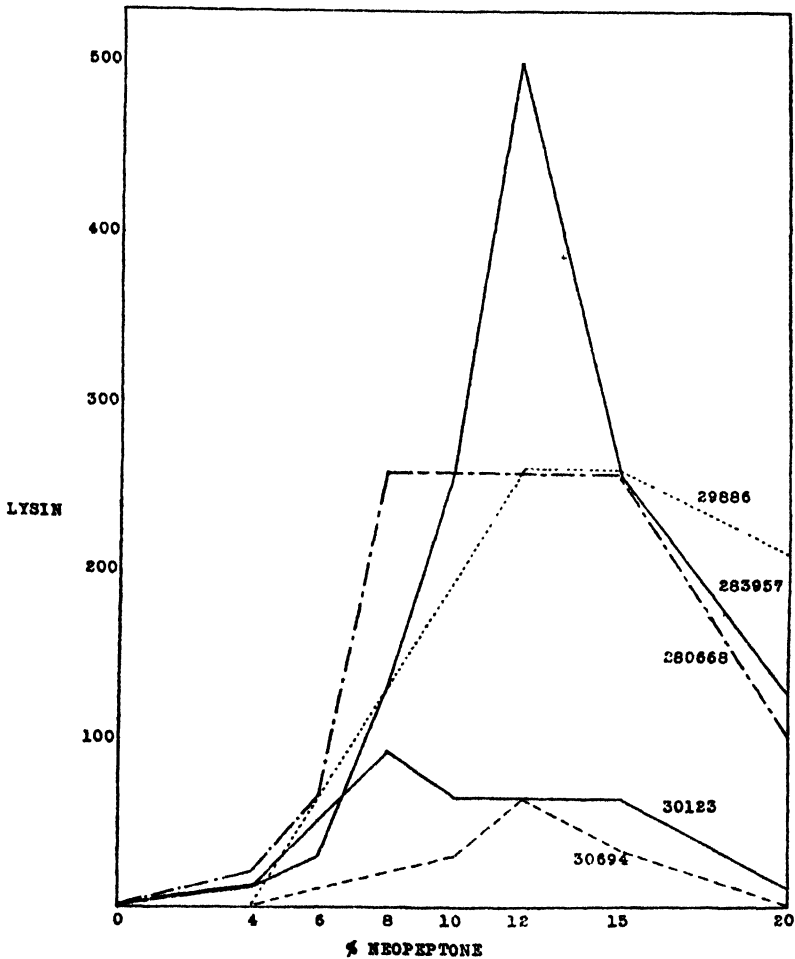


FIG. 1

content of the medium, the titre of lysin was considered to be the volume of culture causing 50 per cent haemolysis of the standard amount of erythrocyte suspension. Tests of five batches of Neopeptone are shown in figure 1.

The most probable explanation of the inhibitory action of high peptone concentration seemed to be the increasing activity of substances present in the digest in amounts too small to be effective in the lower concentrations. Batch 30694 showed the phenomenon most markedly and was examined further.

An aqueous solution of 30694 was dialized against distilled

TABLE 2

## Dialisate

"PEPTONE" FRACTION	CUBIC CENTIMETERS CULTURE							GROWTH
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
<i>per cent</i>								
0	tr.	0	0	0	0	0	0	+
4	1	1	1	tr.	0	0	0	+++
8	4	4	3	2	tr.	0	0	+++
10	3	2	1	0	0	0	0	++
12	0	0	0	0	0	0	0	tr.
20	0	0	0	0	0	0	0	0

## Residue

"PEPTONE" FRACTION	CUBIC CENTIMETERS CULTURE									GROWTH
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	
<i>per cent</i>										
0	tr.	0	0	0	0	0	0	0	0	+
4	4	4	4	4	2	tr.	0	0	0	+++
8	4	4	4	4	4	4	2	tr.	0	++++
10	4	4	4	4	4	4	2	tr.	0	++++
12	4	4	4	4	4	4	2	tr.	0	+++
20	4	4	4	4	4	3	1	tr.	0	++

The relationship between these two fractions and the original peptone in so far as ability to produce streptolysin is concerned is brought out more clearly in figure 2.

water through cellophane for eighteen hours, and the dialisate and residue were evaporated to dryness on a steam bath. The dialisate was yellow in colour, the non-dialisable portion a dark olive-green and the two portions weighed respectively 10.03 and 22.75 grammes. Difco's analysis of Neopeptone shows that material approximately twenty times the weight of free amino acid passed through the cellophane. When these two fractions

were assayed for their ability to promote growth and haemolysin formation (table 2 and fig. 2), it was clear that Neopeptone is separable by dialysis into two portions with very dissimilar properties, and that the inhibitory factor is contained in that portion diffusing most easily through cellophane.

A second dialysis of an aqueous solution of the first dialysate yielded 1.5 grammes of a yellowish resinous substance which on

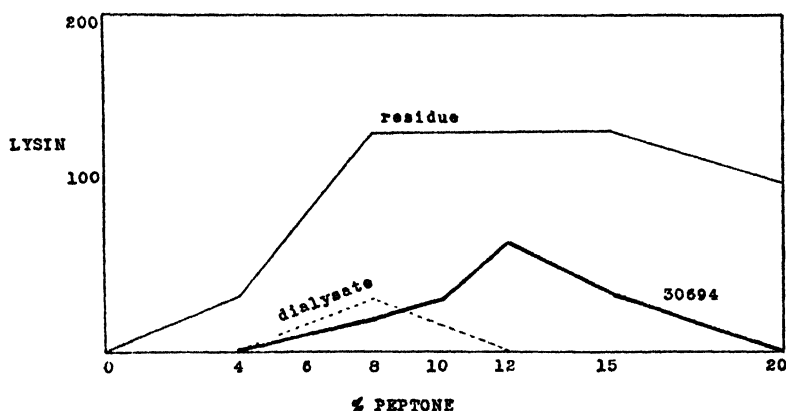


FIG. 2

TABLE 3

"PEPTONE" (2ND DIALYSATE)	GROWTH	HAEMOLYSIN
<i>per cent</i>		
0	+	tr.
2	++	0
5	+++	0
10	±	0
15	0	0

assay as "peptone" in infusion broth yielded the result shown in table 3. Cultivation of an identical set of tubes anaerobically gave identical results.

Extraction of this resinous material with butyl alcohol on a boiling water-bath left a dry white powder (fraction 1). The butyl alcohol solution evaporated to dryness on a water-bath, yielded a white crystalline deposit (fraction 2) in the bottom of



the evaporating dish, and a ring of amber-coloured resinous material (fraction 3) separated on the sides of the dish much as good wine "tears" in a wine-glass. The separation of fractions 2 and 3 was incomplete. Fraction 1 when added to meat infusion functioned as peptone in the sense that it promoted growth of the streptococci, but the resultant culture was practically devoid

TABLE 4

FRACTION 1	CUBIC CENTIMETERS CULTURE							GROWTH
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
<i>per cent</i>								
10	0	0	0	0	0	0	0	—
5	4	4	3	2	0	0	0	+++
2	0	0	0	0	0	0	0	+++
1	0	0	0	0	0	0	0	+++
0.5	0	0	0	0	0	0	0	+++

TABLE 5

FRACTION 2	CUBIC CENTIMETERS CULTURE							GROWTH
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
<i>per cent</i>								
1	4	4	4	2	tr.	0	0	++
0.5	4	4	4	4	2	tr.	0	+++
0.25	4	4	4	4	2	tr.	0	+++
0	4	4	4	4	2	tr.	0	+++
<b>FRACTION 3</b>								
2	0	0	0	0	0	0	0	±
1	4	4	2	1	0	0	0	++
0.5	4	4	4	4	2	tr.	0	+++
0.25	4	4	4	4	2	tr.	0	+++
0	4	4	4	4	2	tr.	0	+++

of haemolytic activity. Such lysin as was formed is almost certainly referable to incomplete separation of the components of the original Neopeptone, and the same reason adequately explains the failure of growth at a concentration of 10 per cent (table 4).

Fractions 2 and 3 did not permit growth when added to meat

infusion. They were therefore assayed by addition in varying concentration to 4 per cent Neopeptone broth from which the inhibitory substances had been removed by dialysis (see Residue, table 2). The yield of haemolysin is shown in table 5.

#### EFFECT OF SERUM

The influence of normal serum on the inhibitory factor was tested by adding 20 per cent of sterile inactivated horse serum to an infusion broth containing 10 per cent of the first dialysate of the original peptone (see table 2). A control with normal saline in place of serum was employed. The resultant cultures titrated for haemolysin are recorded in table 6.

TABLE 6

	CUBIC CENTIMETERS CULTURE										GROWTH
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	
Serum broth.....	4	4	4	4	4	4	3	2	tr.	0	+++
No serum.....	3	2	1	tr.	0	0	0	0	0	0	++±

#### DISCUSSION

Studies like those of Hucker and Carpenter (1927) emphasise the difficulty of achieving protein digests with uniform biological properties, and the fallacy of judging these products on the basis of such factors as the amino-nitrogen content. These workers found that as meat undergoes tryptic digestion the minimum quantity of the digest which will, when added to saline, determine the multiplication of bacteria, increases rapidly during the first few hours of digestion and later decreases again. The value of a digest is not related to its amino-nitrogen content. Considering the probability of some degree of variation in constitution from batch to batch, the general adherence to an arbitrary concentration of 1 or 2 per cent of peptone in culture media is a singular fact. Admittedly most media empirically constructed yield in some measure the desired result, but the fulfilment of special requirements, whether they be certainty of survival of inoculated bacteria or the manifestation of a partic-

ular physiological character, can be materially aided by correct usage of peptone, which is still the most general source of nitrogen for bacteriological purposes.

Extreme concentrations of peptone, whether low or high, predispose to dissociation (Braun and Schaeffer, 1919; Feiler, 1920; de Kruif, 1922). It is a reasonable assumption that between these extremes must lie an optimum at which such digests favour what may be conservatively called the more normal physiological functions, and different functions need not necessarily correspond to the same optimum. Murray and Ayrton (1924) observed that the concentration of a tryptic digest of heart muscle was related in this way to the quantity and virulence of meningococcus growth, and experiments proceeding in this laboratory on the selective cultivation of *Lactobacillus* add weight to the suggestion that the phenomenon has general applicability. As far as haemolytic streptococci are concerned, when untreated peptone is used, the choice of variety and concentration are questions of paramount importance.

Haemolysin formation is not an inevitable accompaniment of streptococcal growth, and even with unfractionated peptone the optimum concentrations for multiplication and lysin are not identical. Manifestly the crude mixture represented by commercial peptone contains substances which separately satisfy the nutritional requirements of haemolytic streptococci in so far as multiplication and formation of haemolysin are concerned. Walbum (1909) regarded peptone as containing an "activator" for a "prolysin" secreted by the organisms. Such a complex system was not required to explain the experimental data, but it did serve to emphasise an early appreciation of the relation between protein digests and bacterial haemotoxins. The components of crude peptone which are essential for haemolysin are to be found in the larger molecules which do not diffuse readily through cellophane, whereas the fraction of smaller molecular weight, possibly peptone in the chemical sense, will promote growth but not lysin. In testing a number of strains of streptococci, there appeared to be a rough correlation between the proteolytic activity of any one strain, and its ability to produce

haemolysin. It is conceivable that the proteolytic enzymes possessed in a varying degree by most Group A haemolytic streptococci may furnish, in nascent form, more readily assimilable products from those fractions of peptone of larger molecular size. Pending more complete chemical separation of the constituents of Neopeptone, the attempt was made to support this hypothesis by using Bactoprotone, which contains more than three times as much proteose as Neopeptone and only one fourth the amount of peptone. The haemolysin yield was markedly reduced. One could agree with O'Meara and MacSween (1936) that a knowledge of the sources and methods of preparation of these commercial digests would help in the understanding of the differences they exhibit. This question of the particular fraction of peptone from which haemolysin is elaborated involves the identity or otherwise of the several lytic agents described. It is not proposed in this place to enter into discussion on this point, but it is pertinent to point out that exhaustion of the Neopeptone broth herein described by growth in it of a Group C haemolytic streptococcus leaves the medium still able to produce haemolysin by a Group A streptococcus. Group A organisms will exhaust the medium for both Group A and Group C streptococci, and all Group A organisms are apparently identical in this respect. This argues in favour of separate lysin precursors for at least two groups of haemolytic streptococci.

As distinct from the fractions which determine bacterial metabolism, commercial peptones contain inhibitory substances which become more evident the higher the concentration of the peptone. Several such substances have been identified, though none of them will individually account for the inhibition.

Dakin (1918) showed that butyl alcohol would effect the separation of the amino acids resulting from tryptic digestion of casein, and Whitehead (1924) found that the butyl alcohol soluble portion of such a digest was inferior to the insoluble fraction, inferring that amino acids exerted an unfavourable effect on the growth of streptococci. Gordon and McLeod (1926) added amino acids to nutrient broth, and found that

certain of them, particularly tryptophane and phenylalanine in 1 per cent concentration inhibited streptococcal growth. It seems clear that the amount of free amino acid in a commercial peptone is insufficient alone to explain the properties of finished peptone broth.

The influence of serum in improving the growth of haemolytic streptococci has been attributed to neutralisation of this deleterious action of amino acids. Yet not only is the amino-acid content of infusion broth a mere fraction of that necessary for growth inhibition, but the addition of serum improves the medium considerable more than the total removal of all inhibitory substances, amino acids included. It would seem therefore that serum acts not only by neutralising the amino-acid effect, but because it adds something the organisms can utilise. This effect of serum is particularly emphasised if judgement is based, not on multiplication of cocci, but on the formation of haemolysin.

Dubos (1930) in fractionating commercial peptones found that the interference with the growth of small inocula of pneumococcus was chiefly referable to a pigment, which appears from the description to be a lipochrome. This pigment was reversibly oxidisable and reducible and inhibited the pneumococcus only in the oxidised state. In the experiments here reported, a substance similar to the one described by Dubos remains in the undialysed non-inhibitory portion of the peptone, and the fraction which interferes with growth and haemolysin formation is not affected by reduction or cultivation under anaerobic conditions.

Others again have attributed the inhibitory properties of peptone to traces of certain metals, particularly copper and iron (O'Meara and MacSween, 1936; Pappenheimer and Johnson, 1936). The amounts of these elements in Neopeptone were not determined, so that their precise importance cannot be stated. The experiments of O'Meara and MacSween, however, on the elimination of copper from nutrient broth, suggest that one virtue of meat extract prepared at 75° to 80°C. resides in the high content of coagulable protein liberated from the meat. The heat coagulation of this protein, subsequent to the addition of peptone, provides the means of removing the inhibitory copper.

The above mentioned authors believe Wright's method (1929, 1933) of cooking the peptone with the meat results in a more satisfactory medium because the copper and iron are removed in the process. In this connection it is of interest that in this laboratory, where the extraction of meat is carried out routinely at 75° to 80°C., it has not been possible to duplicate Wright's results, nor should it be possible if these explanations are correct. It does not seem likely that traces of metals can account for inhibition of streptolysin formation, since the fraction of Neopeptone responsible for this effect is soluble in butyl alcohol. Nevertheless, these desiderata are justified in view of this relatively simple way of eliminating routinely one source of trouble from culture media.

Complete chemical separation of the peptone fractions has not yet been carried out, but the evidence suggests that haemolysin is elaborated from the larger molecules present in protein digests, and that amino acid complexes of smaller size cannot so be utilised even though they will serve to permit increase in numbers of the cocci. The smallest molecules, those which are inhibitory to bacterial life processes, consist of free amino acid, peptic anhydrides and possibly other substances occurring in commercial peptone, sometimes as a result of manufacture in metallic containers. Even without special purification, there is an optimum range over which these digests are most effective.

#### SUMMARY AND CONCLUSIONS

The formation of haemolysin by streptococci is closely related to the variety and concentration of peptone used in the medium. As the peptone concentration is increased the yield of haemolysin increases to a maximum and then decreases. The factors responsible for the inhibitory effect of high peptone concentration are discussed. Peptone fractions can be isolated which separately promote multiplication and haemolysin formation.

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# THE INFLUENCE OF VARIOUS SUBSTANCES AND CONDITIONS UPON STREPTOCOCCAL HAEMOLYSIN<sup>1</sup>

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It has been shown that peptone is the most important single factor in producing high haemolytic activity in cultures of haemolytic streptococci. The purpose of this paper is to demonstrate that the nitrogen metabolism of haemolytic streptococci, at least insofar as haemolysin is one end product, is conditioned by a number of other factors, of which the presence of fermentable carbohydrate is most important.

## THE CARBOHYDRATE FACTOR

Studies of the influence of carbohydrates on protein metabolism date from the observation of Hirschler (1886) that carbohydrate inhibits the formation of indol in culture media. Since that time it has become widely accepted that carbohydrates exert a "sparing action" on nitrogenous substances. Kendall and his collaborators in 1912 initiated a long series of papers on this point. They showed that, from proteins known to contain a carbohydrate fraction, many organisms (among them streptococci) first form acid; and only subsequently may the protein itself be digested to reverse the reaction of the culture. Kendall and Walker (1915) concluded in the case of *Proteus vulgaris* that the effect was due to the suppression by glucose of protease formation. The evidence for or against nitrogen metabolism has not always been satisfactory. De Bord (1923) for instance, points out the fallacy of accepting diminished ammonia production as proof of diminished protein catabolism. In a study of the

<sup>1</sup> This work was aided by a grant from the Banting Research Foundation, Toronto, Ont.

influence of carbohydrate on the nitrogen metabolism of a number of organisms he found the amino-nitrogen constantly increased and the ammonia constantly decreased by the addition of glucose to the medium. In one of the later papers in their series Kendall and Gebauer (1930) showed that *Clostridium welchii* forms toxin best in a medium of glucose content less than 0.2 per cent whereas the histamine-like substance is formed optimally by the same organism in a glucose concentration greater than 2.0 per cent. In this instance one end product sustains and the other contradicts their general thesis that carbohydrate exerts a "sparing action" on nitrogen.

It is generally held that glucose interferes with streptolysin formation either through "protein sparing" or because the acidity developed destroys what lysin is formed (Kuhn, 1912; Stevens and Koser, 1919; Cook *et al.*, 1921). Lyall (1914) states that the interference by carbohydrates is inconstant. These differences are probably referable to the particular media employed by the various workers. In the experiments to be described, fermentable carbohydrate is shown to be an essential component of the medium, without which streptolysin is poorly produced even if suitable nitrogenous foodstuff is available.

#### METHODS

To fresh beef heart infused for two hours at a temperature between 75 and 80°C., 8 per cent of Neopeptone (lot 283957) was added. The mixture was adjusted to pH 7.1 heated ten minutes at 120°C. and filtered through No. 2 Whatman paper. Ingredients were added as required and the finished broth sterilised at 120°C. for twenty minutes.

Media in 5-cc. volumes were inoculated with one drop of a sixteen-hour broth culture of haemolytic streptococcus strain 206, incubated for sixteen hours aerobically, and the whole culture titrated against 0.5 cc. of a 5 per cent rabbit erythrocyte suspension. Titrations were incubated at 37°C. for one hour, and recorded as in the previous paper.

The beef infusion itself contains fermentable carbohydrate, which can be exhausted by a preliminary streptococcal growth.

The titrations in table 1 do not refer to such an exhausted medium. The haemolytic activity of these cultures is not governed by the amount or rate of growth. There is abundant growth in broth containing 1 per cent of glucose, but there is no haemolytic activity.

It is true that glucose, if the pH of the culture falls low enough, is responsible for the acid destruction of the formed streptolysin, but there is no evidence of protein sparing, as will be seen later.

Broth containing 0.5 per cent glucose yields haemolysin up to the tenth hour, but there is rapid destruction on further incubation, once the pH has fallen below 6.0. If, after sixteen hours, the culture is neutralised to pH 7.1, haemolysin again appears

TABLE 1  
*Glucose concentration*

GLUCOSE ADDED	CUBIC CENTIMETERS CULTURE										FINAL pH OF CULTURE
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	
<i>per cent</i>											
0	4	4	4	4	3	2	tr.	0	0	0	7.0
0.1	4	4	4	4	4	3	2	tr.	0	0	6.9
0.2	4	4	4	4	4	4	3	2	1	0	6.8
0.25	4	4	4	4	4	4	3	2	1	tr.	6.7
0.3	4	4	4	4	4	3	2	1	0	0	6.4
0.5	3	2	1	0	0	0	0	0	0	0	5.7
1.0	0	0	0	0	0	0	0	0	0	0	5.2

without significant acid formation during a further sixteen hour incubation. This secondary culture is even more haemolytic if neutralisation is complemented by the addition under sterile conditions of 0.2 per cent glucose.

The importance of glucose in facilitating the metabolism of peptone is seen most clearly in media which can easily become exhausted of glucose during the course of the experiment. A typical experiment is illustrated by figure 1.

The medium contained 0.125 per cent glucose. The content of streptolysin at different times of incubation is represented by the unbroken line. The pH at no time fell below 6.8, a degree of acidity without effect on streptolysin. In fact, lysin is formed

maximally in broth at an initial pH of 6.8. After thirty hours of incubation, four identical cultures were treated respectively (1) by adjustment with sterile N.NaOH to pH 7.1; (2) untreated; (3) adjusted to pH 7.1 and 0.25 per cent sterile glucose added; (4) 0.25 per cent sterile glucose added. The cultures were tested for lysin after incubation for a further fourteen hours.

When the pH of the culture is more alkaline than 6.0, as in this case, neutralisation with sterile NaOH rather depresses

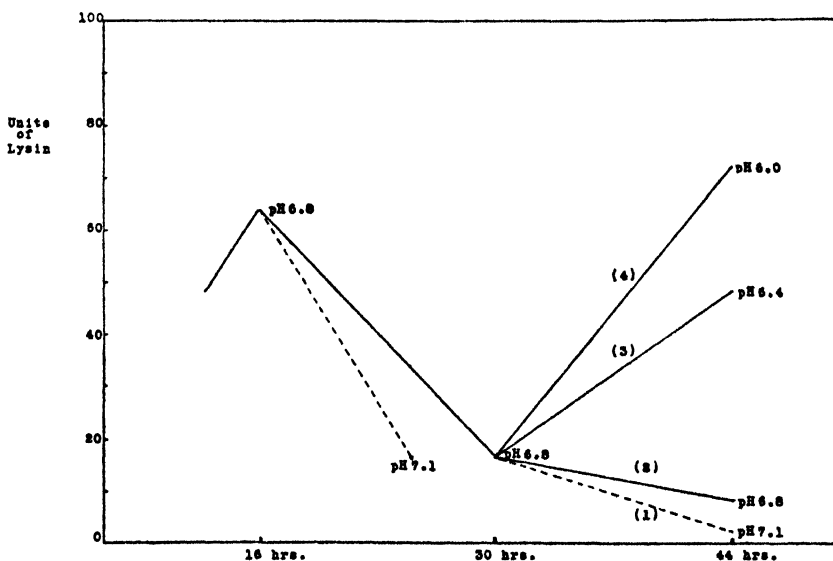


Fig. 1. . . . = streptolysin content if the culture is neutralised to pH 7.1 after incubation for 16 hours and 30 hours respectively. Curves 1, 2, 3 and 4 represent the effect on the lysin of adjusting the culture after 30 hours in the manner described. The pH's express the reaction at the points to which they are appended.

streptolysin, the salt content reaching an unfavorably high level. The addition of glucose, however, with or without neutralisation, fully restores the lytic activity of the culture, even though the final pH falls as low as 6.0. It is evident that broth exhausted of fermentable carbohydrate still contains, by virtue of unused peptone, the potentiality for streptolysin formation provided that glucose be added. The acidity resulting from too much glucose will destroy streptolysin if the pH remains below 6.0,

but neutralisation of the acid, in the absence of fermentable carbohydrate, is in itself insufficient to restore lysin formation by the still viable organisms. In this relation the attempt to provide extra buffering by sodium bicarbonate up to 0.5 per cent was without influence.

The optimum concentration of glucose varies directly as the concentration of peptone. For most batches of Neopeptone these concentrations are 0.25 per cent and 8 to 10 per cent respectively.

#### PROTEIN SPARING

A batch of 8 per cent Neopeptone broth was finished in three portions, to contain respectively 0, 0.125, and 0.25 per cent of glucose. A series of flasks containing 15 cc. were inoculated with *Streptococcus pyogenes* 206 and incubated aerobically. At intervals flasks were withdrawn for the determination of amino-nitrogen, ammonia-nitrogen, pH, colony counts in agar and haemolysin, the first three on centrifuged culture supernatant. The amino-N was determined by Folin's method (1922) and the ammonia-N by Folin's aeration method (1912). Measurements of pH were made colorimetrically. The results of the experiment are shown in table 2 and in figures 2 to 5.

At all times during the period of observation both amino-N and ammonia-N were increased irrespective of the concentration of glucose up to 0.25 per cent. This is not in accord with previous observations. The difference may be due to the use of lower glucose concentrations. Kendall and his co-workers, taking the production of ammonia as a criterion of proteolysis, usually found a decrease in the presence of a carbohydrate. Kendall, Day and Walker (1922) found that glucose increased the total amino nitrogen in cultures of three strains of *Clostridium botulinum*, but caused a decrease in cultures of three other strains. DeBord (1923) with a variety of organisms found that glucose increased the amino-N and decreased the ammonia-N. An examination of the graphs in this latter publication, however, shows that even though the conclusions drawn are valid as the culture ages up to ten days, in some instances the events taking place in the first 24 to 48 hours are not so simple, the curves frequently

transecting each other. Foster (1921) concluded that in cultures of haemolytic streptococcus "the greatest increase in output of ammonia is correlated in a general way with the maximum periods of growth, glucose utilisation, and acid formation. Associated with this increased output of ammonia a corresponding decrease in amino acids is evident." The view of Kendall and Walker (1915) that ammonia formation is the result of

TABLE 2

TIME	GLUCOSE CONCENTRATION	pH	COLONIES	NH <sub>3</sub> -N	AMINO-N	UNITS HAE-MOLTSIN
hours	per cent		millions per cc.	mgm. per 100 cc.	mgm. per 100 cc.	
0	0	7.1	0	16.8	160	0
12	0	7.1	198	20.5	158	100
16	0	7.1	140	23.9	176	75
24	0	7.1	95	25.8	186	5
36	0	7.1	100	32.7	184	2
0	0.125	7.1	0	16.8	156	0
12	0.125	6.8	390	26.6	172	250
16	0.125	6.8	720	31.4	176	350
24	0.125	6.8	290	36.0	182	50
36	0.125	6.8	250	37.0	182	10
42	0.125	6.8	250	29.0	176	5
48	0.125	6.8	260	31.0	181	2
42 + glucose at 36 hours		6.4	430	36.4	180	40
48 + glucose at 36 hours		6.4	400	31.5	189	40
0	0.25	7.1	0	16.8	160	0
12	0.25	6.4	1080	28.0	173	250
16	0.25	6.4	1200	33.3	163	450
24	0.25	6.4	620	21.8	169	100
36	0.25	6.4	470	21.3	184	50

intracellular deaminization of assimilated protein derivatives would explain these changes, as Foster himself points out.

To rest the case for protein sparing on decrease of a substance capable of being metabolised, either amino acid or ammonia, is certainly open to argument. The precise amount present at any given time represents only the balance between production and utilisation. The converse is not open to the same criticism.

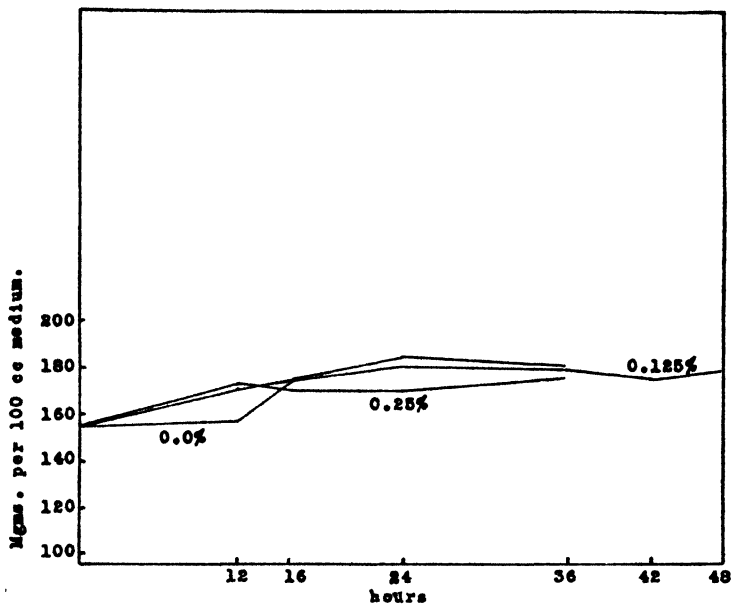


FIG. 2

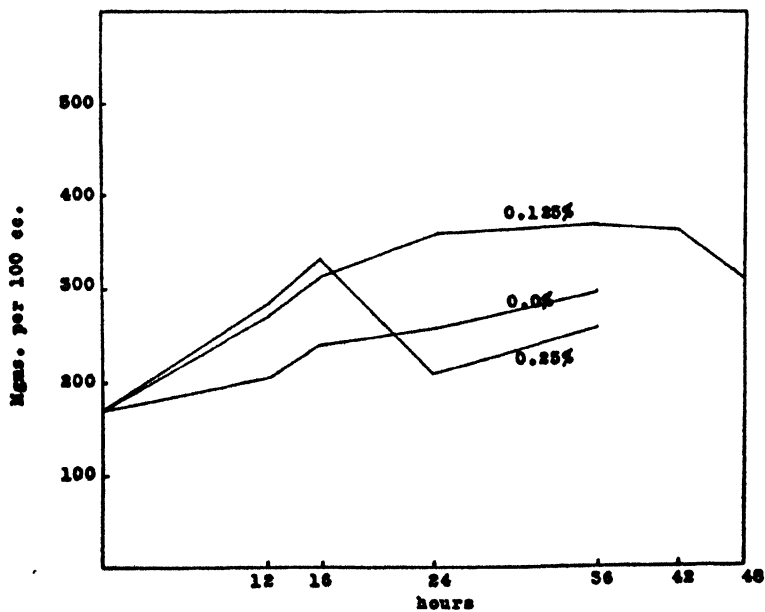


FIG. 3



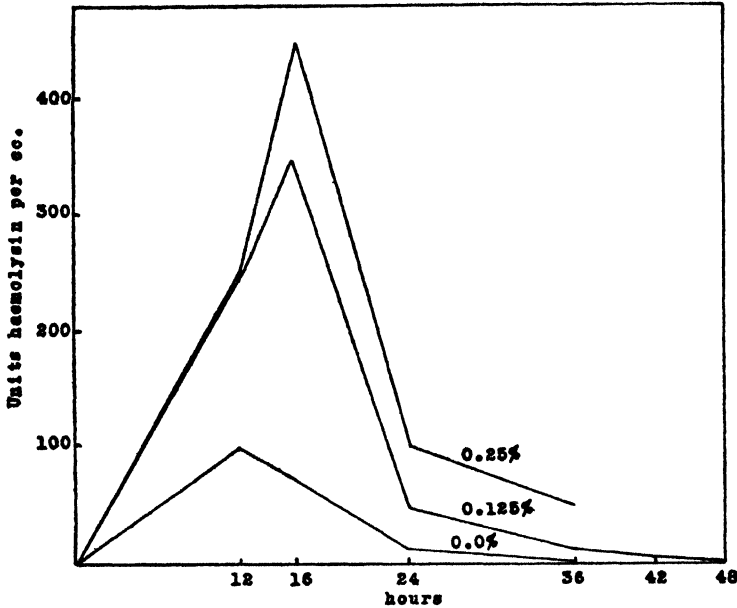


FIG. 4

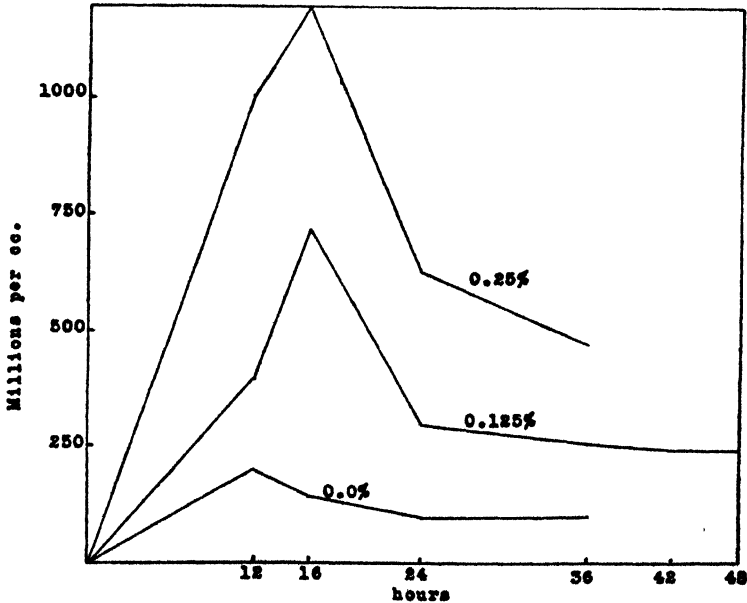


FIG. 5

Whatever the rate of utilisation, increased concentration of amino- or ammonia-nitrogen indicates more proteolysis.

Under the conditions of cultivation used in these experiments haemolytic streptococci utilise nitrogenous foodstuffs more rapidly when glucose is present up to a concentration of 0.25 per cent. Figures 1 and 2 show clearly that in the absence of glucose the rate of increase of amino-N and ammonia-N is definitely slowed. It is significant that 0.25 per cent glucose results in a rapid decrease in this nitrogen after twelve hours, whereas 0.125 per cent glucose broth shows only a slow and late decline. This persistence of utilisable nitrogen in 0.125 per cent glucose broth probably explains why the addition of more glucose to a stable, almost haemolysin-free culture thirty-six hours old results in further proteolysis as evidenced by more haemolysin. There is no such residue of utilisable nitrogen in a culture containing initially 0.25 per cent glucose.

The enormously enhanced formation of new bacterial cells in the presence of glucose is in itself evidence of increased total nitrogen metabolism. The relative importance of viable cells and preformed enzyme is impossible to evaluate, but figures 2, 3 and 5 suggest that the nitrogen metabolism per bacterial cell is not so profoundly modified by these concentrations of glucose as tests on the whole culture would indicate. In this may lie the major justification for insistence on the doctrine of protein sparing in a catabolic sense, but it still does not invalidate the argument that glucose materially assists the total nitrogen metabolism.

Finally, does the glucose influence the formation of haemolysin by modifying the rH of the culture? The fact that neither anaerobic cultivation nor titration of the grown culture by dilution in glucose saline increase the haemolytic titre, suggests that the reducing action of glucose is not a factor.

#### PHOSPHATE

Phosphates were removed from Neopeptone (8 per cent) broth by warming at pH 9.2 and filtering through Whatman No. 2 paper. Varying amounts of  $\text{Na}_2\text{HPO}_4$  were added, the

pH adjusted to 7.0 and sterilisation effected at 120°C. for twenty minutes.

At phosphate concentrations of 0 to 0.5 per cent there was no marked difference in the yield of streptolysin. The adjustment of pH required to precipitate the phosphates in the peptone and meat extract, with its extra manipulations, filtrations and increased salt content, always resulted in a medium inferior to one not so treated. Subsequently media were prepared, therefore, without alteration of the phosphate concentration present in the peptone extract.

It is probable that the high percentage of Neopeptone nullifies the possibility of demonstrating a need for inorganic phosphate, for as Whitehead (1926) points out, the phosphate requirements of haemolytic streptococci are satisfied by organic phosphate, provided this is hydrolysed by subsequent autoclaving. Other workers have similarly demonstrated the importance of phosphorus. Meader and Robinson (1920), using a medium containing serum or tissue extract, came to the conclusion that streptolysin was compounded of organic phosphate and an unstable substance derived from fresh tissue. In their experiments the addition of calcium salts inhibited streptolysin formation by precipitation as calcium phosphate. This effect of calcium was corroborated during the course of the present study.

#### INITIAL pH OF MEDIUM

Beef infusion, 8 per cent Neopeptone, 0.25 per cent glucose, at various pH's was inoculated with strain 206. Cultures were grown for sixteen hours aerobically.

This wide pH range is in general agreement with previous observations (Stevens and Koser, 1919; Cook *et al.*, 1921).

The marked optimum around neutrality and the depressing effect of alkalinisation do not accord with earlier work, and the explanation is probably in the concentration of peptone. In the past it has been customary to regard an initial pH of 7.6 to 8.0 as ideal for streptolysin production, the final pH of the culture being usually less than 6.0. The period in the life of

the culture when lysin is most rapidly increasing in quantity coincides with a pH about 7.0, and the initial alkalinity only postpones eventual cessation of metabolism through accumulated acid. The high peptone concentration acts as a buffer in the same way as does serum, and an initial pH of 7.0 is possible without the ultimate acid inhibition. In addition it furnishes much more of the essential streptolysin precursor than is to be found in the conventional 1 or 2 per cent peptone broth, and this under optimum conditions for its utilisation.

TABLE 3

pH	CUBIC CENTIMETERS CULTURE								
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/512	1/1024
8 0	2	1	0	0	0	0	0	0	0
7 6	4	4	4	4	3	1	0	0	0
7.2	4	4	4	4	4	2	1	0	0
7 0	4	4	4	4	4	2	1	tr.	0
6 8	4	4	4	4	4	3	1	tr.	0
6 4	4	4	4	4	3	2	1	0	0
6 0	4	4	4	2	1	0	0	0	0

TABLE 4

	AGE OF CULTURE IN HOURS										
	1	2	4	7	10	13	16	20	30	48	72
Units of lytic activity....	0	tr.	3	20	150	350	500	250	64	32	6

#### TIME OF INCUBATION

Broth at pH 7.0, containing 8 per cent Neopeptone and 0.25 per cent glucose. This optimum time of about sixteen hours is in accord with the findings of most observers. The variations recorded in an extensive literature as to the time relations of lytic activity are referable to wide variation in the medium employed.

#### GENERAL CONDITIONS AFFECTING STREPTOLYSIN

Colloidal matter suspended in the medium diminished the yield of lysin in proportion to the degree of opacity and dispersion.

This renders necessary the freeing of the broth from all coagulable protein if sterilisation is to be effected by autoclaving. In attempts to demonstrate neutralisation of the lysin by immune sera, it has been apparent that opalescent sera owed something of their activity to the influence of the suspended matter. Similarly the attempt to make a semi-solid medium by the addition of small amounts of agar considerably diminishes the lytic activity of the culture.

Anaerobic cultivation gives slightly inferior results, although the period of time during which lysin is demonstrable is considerably increased. This latter is not due to lessened acid production in the culture, but apparently in part to the removal of one damaging factor—oxidation. The addition of 5 to 10 per cent carbon dioxide to the atmosphere in which the culture is grown decreases the lytic potency. The effect is probably a specific one, for the change in pH produced by carbon dioxide in the atmosphere is insufficient to account for the loss of haemolysin.

If the virulence of a strain of *Streptococcus pyogenes* is raised by animal passage, the haemolytic activity of the resultant culture is demonstrably increased. This fact has been noted by others (Cook *et al.*, 1921) and might be correlated with the observation of Rosenthal and Patai (1914) that virulent strains are more proteolytic than avirulent strains.

#### COMMENT

Cultures of haemolytic streptococci in the serum-free medium used in these experiments are characterised by very high haemolytic activity. While for reasons set forth in the examination of the properties of peptone the requirements for maximum growth and maximum lysin are not identical, nevertheless this high peptone broth does promote the early and maximal multiplication of haemolytic streptococci. A comparison of this medium with the standard laboratory broth and a commercial nutrient broth on their ability to promote multiplication of haemolytic streptococcus strain 206, together with the minimum number of these organisms capable of initiating growth in 5 cc. of these media is given in table 5.

This Neopeptone broth has served to emphasise the physiological differences between the various members of the genus streptococcus. For instance, *Streptococcus viridans* fares so poorly on this medium that the occasional culture difficult to classify on blood plate appearances can thereby be excluded from confusion with *Streptococcus pyogenes* with as great a certainty as by testing for haemolysin.

The conditions which underlie escape of the streptolysin from the bacterial cells have not been established, nor is it certain that the antigenic filtrable streptolysin described by Todd (1932) is identical with the various lytic agents involving the use of serum, either in the culture fluid or in the extraction of the grown cocci. The medium here developed gives an extraordinarily high yield of streptolysin, but it remains quantitatively

TABLE 5

MEDIUM	6 HOURS	8 HOURS	10 HOURS	24 HOURS	MINIMUM NUMBER OF <i>S</i> STREPTOCOCCI
Experimental.....	$100 \times 10^6$	$600 \times 10^6$	$1500 \times 10^6$	$3000 \times 10^6$	1
Standard.....	0	tr. turbid	$200 \times 10^6$	$600 \times 10^6$	50
Commercial...	0	0	0	0	Infinity

associated with the organisms. Despite the fact that no lysin can be detected in filtrates or centrifugates, it is liberated almost entirely by shaking the washed organisms in serum for three minutes. These properties will be more fully dealt with subsequently. There is no evidence of the formation in this medium of filtrable haemolysin. Attempted reduction of filtrate or whole culture with sodium hydrosulphite always results in rapid destruction of the haemolysin.

#### CONCLUSIONS

In the low concentrations of glucose and high concentrations of suitable peptone employed, there is no evidence that the carbohydrate spares the peptone. There is a decided stimulating effect.

In highly buffered media the optimum pH for haemolysin formation is in the region of 7.0.

A number of other factors influencing haemolysin formation are discussed.

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# THE CLASSIFICATION OF ACID-FAST BACTERIA

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During the last fifty years acid-fast organisms, other than tubercle bacilli, have been cultivated from many sources. With few exceptions, these organisms grow readily and rapidly on ordinary media, especially when glycerol is present. These organisms are non-pathogenic in the usual sense although large doses ordinarily produce abscesses and, sometimes, death of experimental animals.

These organisms, without exception, have been so meagerly described that it is not possible to recognize them. As a consequence the literature contains references to a considerable group of organisms which are known by the name of the person who isolated them, or by the source from which they came. The descriptions lead one to suspect strongly that many of these organisms, coming from diverse sources and known under different names, really belong to a single species.

During the last few years we have isolated a large group of acid-fast organisms from soil. Having these on hand, and also a group of strains isolated by others from various sources, we have endeavored to find means of identifying and classifying them. Both cultural and serological methods have been used. In this report we shall deal only with the cultural work.

Growth at 47°C., which was first used by Thomson (1932) as a means of separation of the saprophytic mycobacteria, forms a major basis of the classification of this group. This criterion has been applied to a much larger collection, and other means of confirming the grouping have been found. Some progress has been made, apparently, in breaking down the three main groups into



sub-groups, several of which appear to consist of a single species. On the other hand, about 20 per cent of our strains do not fit well into any of the larger groups and certainly require further study. It may be said in passing that the great majority of our strains have been isolated from soil by means of Söhnngen's (1913) technique which utilizes the ability of these organisms to obtain metabolic carbon from paraffin. It is quite possible that some of the types that are not common in our collection would be much more numerous if other materials had been examined. It is of considerable interest to note, however, that many organisms isolated from human and animal tissues also utilize paraffin and in other ways appear to be identical with strains that have come from soil.

Two hundred and fifty-two strains of saprophytic acid-fast bacteria, including those described by Thomson, have been studied. These strains were isolated from soil (212), bovine (8), avian (1), and porcine (1) tissues, human tissues (9), and the tissues of cold-blooded animals (5). The human series include a number that were isolated from the lesions of leprosy and others that had no relation to this disease. All of the soil types, with but six exceptions, have been isolated in this laboratory by Frey and Hagan (1931), Willie (1934) and Gordon and Hagan (1937). Likewise, a few types of animal origin have been isolated by us. The other strains have been received from workers elsewhere and from culture collections, principally those of the Lister Institute of London and the American Type Culture Collection of Chicago. We cannot vouch, therefore, for the authenticity of all of our named strains but can only say that all of these appear to agree with the original descriptions so far as we have been able to find and interpret them. It is probably safe to conclude that the majority of these strains are true to name.

#### EXPERIMENTAL METHODS

All of our strains have been subjected to a series of cultural tests in an attempt to find criteria for their differentiation. The ones which have given useful results are described below.

(a) *Gross appearance.* A simple inspection of the cultures

growing on glycerol agar slopes is enough to convince anyone that they are not all alike. Since these differences are difficult to describe accurately, we do not propose to try to do so. We have not used the gross appearance of the cultures as a means of differentiation, but have always been careful not to place in a group, otherwise formulated, strains that were not identical in appearance and behavior. There has been only one departure from this rule; in the case of strains that dissociate readily from the S to R form. Many of the saprophytic acid-fast organisms that usually occur in the S form dissociate easily and frequently. We have included R forms with the normal S forms only when we have compared them with variants that we have developed from strains which normally occur as S and have found them to be identical in appearance.

(b) *Growth at 47°C.* Glycerol-phosphate agar slants were inoculated, partially sealed with notched corks, warmed to 47°C. in a water bath, then incubated at this temperature for two weeks. Growth or absence of growth was readily determined.

(c) *Survival at 60°C. for one hour.* Three cubic centimeters of ten days' old cultures in glycerol-phosphate broth were pipetted into 5 cc. ampoules. The ampoules were sealed, fastened in a wire rack, and immersed in a 60°C., constant temperature water bath for one hour. At the end of this time the ampoules were cooled, their tips broken, and the contents pipetted to glycerol-phosphate agar slants. If growth appeared on the slopes, it was compared macroscopically and microscopically with the original culture.

(d) *Growth on Dorset's synthetic medium without glycerol.* A synthetic, asparagin medium was prepared according to the formula given by Dorset (1926), except for the omission of 7 per cent glycerol and the addition of 1.5 per cent agar. The cultures were inoculated on slopes of this medium and incubated at 37°C. for two weeks.

(e) *Utilization of carbohydrates.* The ability of these cultures to utilize certain carbohydrates as the sole source of carbon was determined by the method described by Merrill (1931). The medium has the following composition:

	per cent
NaCl.....	0 5
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	0 5
MgSO <sub>4</sub> .....	0.0005
KH <sub>2</sub> PO <sub>4</sub> .....	0.04
K <sub>2</sub> HPO <sub>4</sub> .....	0 16
Agar .....	2 0
Phenol red (in alkaline solution).....	0 0001

Five cubic centimeters of the above medium were placed in tubes and sterilized by autoclaving. One-half per cent of the carbohydrate, which had been sterilized by Berkefeld filtration, was added to each tube.

Utilization of each carbohydrate was established by growth of the culture on the above medium and by the accompanying acid color of the indicator.

(f) *Action in litmus milk.* The cultures were inoculated into litmus milk and incubated at 25°C. for one month.

(g) *Nitrate reduction.* The cultures were grown in a beef-extract medium containing 1 per cent KNO<sub>3</sub>. After ten days' incubation at 25°C. they were tested for nitrites with Trommsdorf's reagent.

## RESULTS

Following the primary grouping of Thomson, we have placed our cultures in three groups. In group I all but four strains consist of the *Mycobacterium smegmatis* type; in group III all but one strain is of the *Mycobacterium phlei* type. Group II is a more heterogenous one, although 66 of the 95 strains appear to belong to a single species, about equally divided between the R and S forms. The other 29 strains, although having many features in common, are evidently not identical. Among these 29 strains are most of our so-called lepra strains, also most of the tubercle bacilli of cold-blooded animals.

The cultures have been grouped according to the following plan:

### Group I

Fails to survive 60°C. for one hour.

Grows at 47°C.

a. Utilizes arabinose.

105 strains similar in appearance and cultural reactions.

b. Unable to utilize arabinose.

4 strains different in appearance and cultural reactions from above cultures and from each other.

*Group II*

Fails to survive 60°C. for one hour.

Does not grow at 47°C.

a. Unable to utilize sorbitol.

1. Unable to utilize arabinose.

66 strains similar in appearance and cultural reactions.

22 strains different from above cultures and from each other in appearance and cultural reactions.

2. Utilizes arabinose.

7 dissimilar strains.

b. Utilizes sorbitol.

18 dissimilar strains.

*Group III*

Survives 60°C. for one hour.

Grows at 47°C.

a. Utilizes arabinose.

29 strains similar in appearance and cultural reactions.

b. Unable to utilize arabinose.

1 strain differing in appearance and cultural reactions from above cultures.

The cultural features and grouping of our cultures are indicated in table 1.

*Group Ia.* The 105 strains assembled in this division grow rapidly at 25°C. and 37°C. In early stages, on glycerol agar slants, the growth is dull, creamy white, spreading and translucent. After three or four days the growth becomes thickened, wrinkled and opaque (see fig. 1). After three or four weeks at room temperature a deep yellow or orange color develops. This color change is quite characteristic of this group since it was observed in only two cultures not included in this division.

*Group Ib.* Four other cultures were included in group I because they grow at 47°C. and do not survive 60°C. for one hour, although they are dissimilar to each other and to members of group Ia in appearance and in several cultural reactions. Two soil strains are creamy white and do not change color with aging; one is of the smooth type and produces a smooth, unwrinkled

growth; the other is rough in type and presents a waxy, deeply wrinkled appearance. The third culture in this group, an isolation from a case of human nodular leprosy, is rough in type and yellow in color, resembling *Mycobacterium phlei* in appearance. The fourth strain, *Mycobacterium leprae* I of Clegg, is salmon pink in color and produces a smooth growth with nodular elevations

TABLE 1  
*Grouping of saprophytic, acid-fast cultures*

GROUP	NUMBER OF CULTURES	SURVIVAL AT 60°C. FOR 60 MINUTES	GROWTH AT 47°C.	GROWTH ON MERRILL'S MEDIUM WITH VARIOUS CARBON SOURCES							GROWTH ON DORSET'S SYNTHETIC MEDIUM, NO GLYCEROL	ALKALI PRODUCTION IN LITMUS MILK	NITRATE REDUCTION
				Sorbitol	Arabinose	Galactose	Trehalose	Mannitol	Fructose	Sucrose			
Ia	105	—	+	+	+	+	+	+	+	—	—	+	101+
Ib	4	—	+	±	—	±	+	±	+	—	—	+	±
IIa <sub>1</sub>	66	—	—	—	—	—	±	±	+	—	—	+	±
	22	—	—	—	—	—	±	±	19+	19—	—	19+	±
IIa <sub>2</sub>	7	—	—	—	+	—	±	±	3—	3+	—	3—	±
									6—	1+		6+	
												1—	
IIb	18	—	—	+	±	±	±	±	+	±	—	+	±
IIIa	29	+	+	+	+	+	+	+	+	—	±	+	+
IIIb	1	+	+	s1	—	s1	+	+	+	—	—	s1	+

Total number of cultures—252.

which tend to coalesce to form ridges. The cultural variations may be seen in table 1.

*Group IIa<sub>1</sub>*. Eighty-eight strains are incapable of utilizing either sorbitol or arabinose as sources of carbon. Of these 88 cultures, 66 are sufficiently alike in appearance and bio-chemical reactions to indicate their close relationship. These 66 strains produce a creamy white growth which does not change color with

aging. The growth is not quite as rapid, spreading, or heavy as that presented by members of group Ia. Thirty-five of the 66 strains are rough in type and produce a crumbly, piled-up growth as shown in figure 2. The remaining 31 cultures are smooth in



FIG. 1. *M. SMEGMATIS*, SHOWING TYPICAL APPEARANCE OF CULTURES OF GROUP Ia

type, and their growth resembles a streak left by a well-filled paint brush. (See fig. 3.) Sixty-five of these cultures were obtained from soil; the remaining one is *Mycobacterium ranae* of Kuster. The 22 strains arbitrarily included in this division because of their inability to attack sorbitol and arabinose are, for

the most part, dissimilar in appearance and in other bio-chemical features. Five creamy white strains, *Mycobacterium chelon* (smooth type), *Mycobacterium schlangen* (smooth), the bacillus of Bayne-Jones (smooth), the bacillus of Hastings (rough), and



FIG. 2. SOIL ISOLATION, SHOWING TYPICAL APPEARANCE OF ROUGH STRAINS INCLUDED IN GROUP II<sub>R</sub>

one soil isolation (smooth), utilize none of the carbohydrates. Nine deep yellow, smooth, soil cultures ferment trehalose, mannitol and fructose. Three strains, *Mycobacterium leprae* of Duval and two soil isolations (one a smooth yellow and the other a rough, creamy white), obtain necessary carbon from trehalose, mannitol,

fructose, and sucrose. The remaining five strains, *Mycobacterium thamnophicus*, Plum's bacillus (rough, pink), and three soil strains (one a rough yellow and two smooth yellow), vary considerably from each other and from the above groups.



FIG. 3. SOIL ISOLATION SHOWING TYPICAL APPEARANCE OF SMOOTH STRAINS INCLUDED IN GROUP  $IIa_1$ .

*Group  $IIa_2$ .* Seven cultures were separated from the preceding 88 strains because of their ability to ferment arabinose. Their variations in pigment, type, and cultural features are recorded at top of next page.



NAME OR SOURCE	TYPE	PIGMENT	GALACTOSE	TREHALOSE	MANNITOL	FRUCTOSE	SUCROSE
<i>M. marimum</i>	Smooth	Deep yellow	-	-	-	+	-
Bovine lymph gland	Smooth (slimy)	Yellow	-	+	+	+	-
Soil	Smooth	Deep yellow	-	+	+	+	-
Soil	Smooth	Deep yellow	-	-	+	+	-
Soil	Smooth	Yellow	-	-	+	+	-
Soil	Smooth	Yellow	-	+	+	+	+
Soil	Smooth	Creamy white	+	+	+	+	-



FIG. 4. *M. phlei*, SHOWING TYPICAL APPEARANCE OF ROUGH CULTURES INCLUDED IN GROUP IIIa

*Group IIb.* This sub-group, including those strains which utilized sorbitol, was of interest because it contained five cultures bearing the name *Mycobacterium leprae*. One smooth yellow leprae strain ferments only sorbitol and fructose. Two cultures, *Mycobacterium leprae* (Lister collection No. 518), and the leprosy

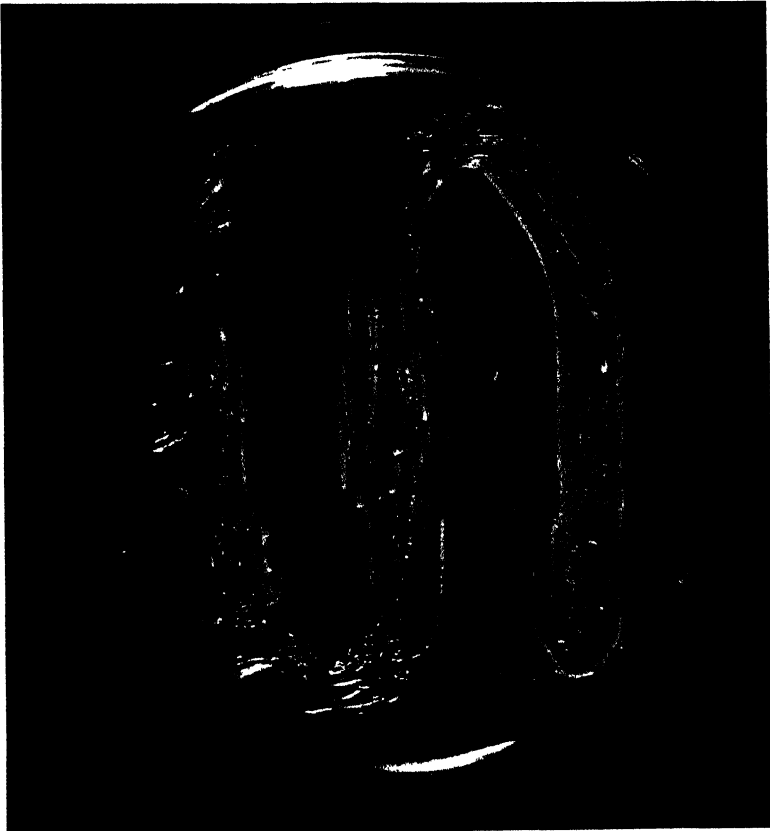


FIG. 5. SOIL ISOLATION. SHOWING TYPICAL APPEARANCE OF STRAINS INCLUDED IN GROUP IIIa

bacillus of Elly, produce a yellow, crumbly, piled-up growth and utilize sorbitol, trehalose, mannitol and fructose. The same carbohydrates are attacked by two other smooth, salmon pink leprae cultures (Brinkerhoff II and Clegg II), a salmon pink strain isolated from a calf skin lesion, and by three rough, creamy white

TABLE 2  
*Saprophytic acid-fast cultures included in various groups*

GROUP	NUM- BER OF CUL- TURES	NAME OF CULTURE	SOURCE	ISOLATED BY
Ia	1	M. smegmatis	Smegma	Alvarez and Tavel
	2	M. graminis	Plant dust	Moeller
	2	M. stercoris	Cow manure	Moeller
	1	M. berolinense	Butter	Rabinowitch
	1	M. paratuberculosis		Briot
	1	M. pseudoperlsucht		
	1	Grassberger's bacillus	Butter	Grassberger
	1	Bacillus aus Nasenchleim	Nasal exudate	Karlinski
	1	Traum's bacillus	Bovine lymph gland	Traum
	94	Unnamed	Soil	Cornell University
Ib	1	M. leprae I	Human leprosy	Clegg
	1	Kat no 352	Human leprosy	Carpenter*
	2	Unnamed	Soil	Cornell University
IIa <sub>1</sub>	1	M. ranae	Frog's liver	Kuster
	1	M. thamnophis	Garter snake	Aronson
	1	Bayne-Jones acid-fast	Human lung	Bayne-Jones
	1	M. chelonae	Turtle's lung	Friedmann
	1	M. schlangen	Snake	Sibley
	1	M. leprae	Human leprosy	Duval
	1	Plum's bacillus	Milk	Plum*
	1	Guernsey heifer acid-fast	Bovine tissue	Hastings
	80	Unnamed	Soil	Cornell University
IIa	1	M. marmum	Fish	Aronson
	1	Lymph gland acid-fast	Bovine lymph gland	Hastings
	5	Unnamed	Soil	Cornell University
IIb	1	M. leprae		Lester Institute no 518
	1	M. leprae		Lilly
	1	M. leprae	Human leprosy	Brinkerhoff
	1	M. leprae	Human leprosy	Clegg
	1	Unnamed	Skin lesion of calf	Dames
	1	Wong	Human leprosy	Carpenter*
	12	Unnamed	Soil	Cornell University

\* These cultures were sent to us by these workers, but, so far as is known, have not been described in the literature

TABLE 2 *Concluded*

GROUP	NUMBER OF CULTURES	NAME OF CULTURE	SOURCE	ISOLATED BY
IIa	2	M. phlei	Timothy grass	Moeller
	1	Unnamed	Hen's spleen	Cornell University
	2	Unnamed	Bovine lymph glands	Cornell University
	1	Zeissig's bacillus		Zeissig*
	22	Unnamed	Soil	Cornell University
	1	Unnamed	Bovine skin lesion	Cornell University
IIb	1	Hog skin bacillus	Hog's skin	Buckley

soil isolations. Three red pigmented strains which utilize sorbitol, trehalose, mannitol, fructose and sucrose, were also included. The remaining six, dissimilar cultures (two smooth, yellow; two smooth, creamy white; and two smooth, creamy white which change to deep yellow with age), are soil strains which utilize all sugars except sucrose.

*Group IIIa.* The 29 cultures assembled in this group are deep yellow or orange in color regardless of age. Eighteen are rough in type and produce a rapid, spreading, waxy, coarsely wrinkled growth (see fig. 4). The eleven smooth strains present a smooth, spreading, mucoid growth as shown in figure 5. These strains were separated from those of group Ia because of their ability to resist 60°C. for one hour and because of their different appearance. All of the rough strains and a few of the smooth ones grow on Dorset's synthetic medium which contains no glycerol.

*Group IIIb.* One culture, the hog-skin bacillus of Buckley, was assigned to this group because of its ability to withstand 60°C. for one hour and to grow at 47°C. It is rough in type and presents a pale yellow, crumbly growth.

The names and sources of our cultures and their disposition into the various groups are shown in table 2.

In our search for criteria for separating our cultures several tests not employed in this classification were made. Although some of them gave no useful information, others may be used later for further subdivision of these and other cultures. None of the cultures tested liquefy gelatin, form indol, or utilize raffinose.

All strains produce catalase, grow at pH 9.0 and utilize glucose and glycerol. Some of the cultures give positive results and some negative to the following tests: ammonia production from peptone, starch hydrolysis, agglutination by concanavalin A, growth at 5°C., survival of 60°C. for ten minutes, growth in high salt concentrations, acid production in Dorset's synthetic medium, actual amount of glucose utilized by cultures in glucose broth, final pH in glucose broth and utilization of the following substances for metabolic carbon: maltose, dulcitol, salicin, inositol, sodium citrate, ammonium lactate and phenol.

#### SUMMARY

The cultural reactions of a collection of 252 saprophytic, acid-fast cultures from soil, plants, human and animal tissues and secretions have been studied in an endeavor to find standards for the classification of the saprophytic mycobacteria. Eighty per cent of the strains were readily separated into three groups of closely related cultures. The remaining 20 per cent of the strains, which differed from the above and in most cases from each other, were arbitrarily distributed among the three major groups as sub-groups.

The counsel and assistance of Dr. W. A. Hagan in conducting and presenting this study are gratefully acknowledged.

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# THE IDENTITY OF "BACILLUS INNUTRITUS" (KLEINSCHMIDT) AND BACILLUS PARAPUTRIFICUS (BIENSTOCK)

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From time to time, over a period of many years, we have recovered from the feces of infants and children, from fecally-contaminated surgical wounds, from a decubitus ulcer, and from post-mortem cultures from the heart blood, peritoneal and pleural fluids in adults, a species of anaerobic bacillus whose close resemblance to the "*Kopfchenbakterien*" of Escherich was finally pointed out by Hall and O'Toole (1935) and whose identity with the *Bacillus paraputrificus* of Bienstock was shown by Hall and Snyder (1934).

In 1934, Kleinschmidt of Cologne, Germany, reported an anaerobic bacillus occurring in the stools of infants at periods of malnutrition, which in his opinion differed from previously described bacteria and which he called "*Bacillus innutritus*."

The description of "*B. innutritus*" was at once suggestive of *B. paraputrificus*, and it so happened that the senior author was able personally to visit Dr. Kleinschmidt in the *Kinderklinik der Stadtliche Krankenanstalt Lindenberg* and to secure two strains of "*B. innutritus*" for comparison. These were numbered 9340 and 9341 in our series and compared morphologically and culturally with our strains 4042Ea and 4453 of *B. paraputrificus*, isolated by Miss O'Toole from the stools of three- and four-day old infants in 1930.

It is unnecessary to repeat all of the details of our findings which showed definitely that all of these strains belonged to the species, *Bacillus paraputrificus*, as described by Hall and Snyder.

We again observed the variations in Gram-staining in the coagulation of powdered milk solution and in action on sterilized fresh milk as already recorded.

We confirmed all of the morphological and cultural observations previously recorded except as to the fermentation of raffinose, which, we found, was not fermented, either by the above four strains or by any of the additional sixteen strains previously studied. Consulting Snyder's thesis, we found that in the original observations raffinose was not fermented; raffinose was wrongly included by Hall and Snyder among the fermented carbohydrates.

In addition, we tested arabinose, rhamnose, dulcitol, and inositol which were not fermented.

We likewise found the first four strains mentioned above to be essentially non-pathogenic by subcutaneous inoculation in rabbits and guinea pigs although a slight transient oedema was observed in guinea pigs, which always subsided without necrosis.

Aside from the above proofs of identity, the most conclusive evidence was found in the serological tests.

#### SEROLOGY

Desiring to avoid the non-specific acid agglutination described by Snyder in his serological studies on *Bacillus paraputrificus* (1936), we immunized rabbits with cultures grown in 1 per cent glucose broth in constricted tubes with marble seals, intending to neutralize such cultures for the actual tests as advised by Snyder. We observed, however, that it was difficult to avoid overneutralization; there was a tendency for the cultures to become clear, owing apparently to solution of the bacteria, so that they became worthless for agglutination tests. We then attempted to use cultures grown in meat-infusion broth without added sugar, as also advised by Snyder, but the growth was too sparse. We then decided to use meat-infusion broth with 0.2 per cent glucose in which *B. paraputrificus* was found to grow abundantly but without excessive production of acid.

Snyder found acid agglutination to begin with *B. paraputrificus* at pH 5.1 while the terminal acidity in 1 per cent glucose broth

cultures at forty-eight hours was pH 4.4 to pH 4.8. With the assistance of Mr. Nicholas Duffett in the electrometric determination, we found pH values of 4.64 in 1 per cent glucose and of 5.37 in 0.2 per cent glucose broth after forty-eight hours of growth; therefore, it was possible to use this latter medium and still avoid acid agglutination.

Two rabbits weighing about 2 kgm. each were immunized. Cultures were prepared two days ahead of time for each injection. All inoculations were made subcutaneously, using 2 cc. of living culture each time.

Rabbit 3028 was inoculated with "*B. innutritus*" 9340 on December 14, 18, 22, 28, 31, 1936, January 4, 8, 12, 15, 19, 22, and 27, 1937. There was no apparent loss of weight and no appearance of adverse symptoms or lesions during treatment. Five cubic centimeters of blood were drawn from the ear vein on February 3, 1937. A preliminary test showing the titre of the serum to be satisfactory, 25 cc. of blood were drawn from the left ventricle of the heart on February 4, 1937. This serum was used in the final tests.

Rabbit 3027 was injected with *B. parapatrificus* 4042Ea December 14, 18, 22, 26, 1936, January 4, 8, 12, 15, 19, 22, 27, and 30, 1937. No lesions or adverse symptoms were observed during this period. On February 9, 1937, 5 cc. of blood were obtained from an ear vein. The titre of the serum proving satisfactory, 25 cc. of blood were secured by cardiac puncture on February 10, 1937.

Agglutination tests were made against all of the strains of *B. parapatrificus* available and the two strains of "*B. innutritus*," using cultures in 0.2 per cent glucose broth incubated for forty-eight hours, diluted with saline solution to about 500 million organisms per cubic centimeter. The tests were incubated at 37.5°C. for three hours, recorded, reincubated for three hours more, again recorded, and then left at room temperature for the remaining eighteen hours, and finally recorded. There was never any evidence of contamination under these conditions. The results of the agglutination tests are shown in tables 1 and 2.

Table 1 shows that the serum prepared against "*B. innutritus*"



TABLE 1

*Agglutination tests with serum of rabbit 3028 immunized against "Bacillus innuitus" 9340*

CULTURE NUMBER*	TIME IN HOURS	DILUTIONS OF SERUM										Control
		20	40	80	160	320	640	1280	2560	5120	10240	
491	3	-	-	-	-	-	-	-	-	-	-	-
	6	++	++	+	-	-	-	-	-	-	-	-
	24	++++	++++	++++	++	-	-	-	-	-	-	-
1538	3	+++	+++	+++	++	++	++	+	-	-	-	-
	6	++++	++++	++++	+++	+++	+++	++	-	-	-	-
	24	++++	++++	++++	+++	+++	+++	++	+	+	-	-
3572Ae	3	+	-	-	-	-	-	-	-	-	-	-
	6	++	++	++	+	-	-	-	-	-	-	-
	24	++++	++++	++++	++++	-	-	-	-	-	-	-
3945	3	++	++	+	+	-	-	-	-	-	-	-
	6	+++	+++	+++	+++	++	+	+	-	-	-	-
	24	++++	++++	++++	++++	+++	+	+	-	-	-	-
3953	3	+++	+++	++	++	++	+	+	+	-	-	-
	6	+++	+++	+++	+++	++	++	+	+	+	+	-
	24	+++	+++	+++	+++	++	++	+	+	+	+	-
3978B	3	+++	+++	+++	+++	++	+	+	-	-	-	-
	6	++++	++++	++++	++++	+++	+++	+++	++	+	-	-
	24	++++	++++	++++	++++	+++	+++	+++	++	+	-	-
4013Cb	3	++	++	++	+	-	-	-	-	-	-	-
	6	+++	+++	+++	++	+	+	+	-	-	-	-
	24	++++	++++	+++	+++	++	++	+	-	-	-	-
4042Ea	3	+	+	-	-	-	-	-	-	-	-	-
	6	++	++	+	-	-	-	-	-	-	-	-
	24	++++	+++	+++	+	+	-	-	-	-	-	-
4065Bb	3	++	++	+	-	-	-	-	-	-	-	-
	6	+++	+++	++	+	-	-	-	-	-	-	-
	24	++++	++++	+++	+++	+++	++	++	-	-	-	-
4104	3	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-
	24	+	+	-	-	-	-	-	-	-	-	-
4351	3	++	++	+	-	-	-	-	-	-	-	-
	6	+++	+++	++	+	-	-	-	-	-	-	-
	24	++++	++++	+++	+	-	-	-	-	-	-	-
4398Cb	3	++	++	+	-	-	-	-	-	-	-	-
	6	+++	+++	+	-	-	-	-	-	-	-	-
	24	++++	+++	++	-	-	-	-	-	-	-	-

\* Cultures numbered 491 to 9193 were the original strains of *B. paratyphicus* of Hall and Snyder; cultures numbered 9340 and 9341 were Kleinschmidt's strains of "*B. innuitus*." Culture 9340 was the one used in preparing the serum.

TABLE 1—*Concluded*

CULTURE NUMBER*	TIME IN HOURS	DILUTIONS OF SERUM										Control
		20	40	80	160	320	640	1280	2560	5120	10240	
4453	3	—	—	—	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—	—	—	—
	24	+++	+++	+++	++	++	—	—	—	—	—	—
5465	3	++	++	++	+	+	+	—	—	—	—	—
	6	+++	+++	+++	++	++	+	—	—	—	—	—
	24	+++	+++	+++	++	++	+	—	—	—	—	—
8861	3	+	—	—	—	—	—	—	—	—	—	—
	6	+++	+++	++	+	+	—	—	—	—	—	—
	24	++++	++++	+++	+	+	—	—	—	—	—	—
8862	3	—	—	—	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—	—	—	—
	24	+++	++	++	+	—	—	—	—	—	—	—
8863	3	—	—	—	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—	—	—	—
	24	+	+	—	—	—	—	—	—	—	—	—
9103	3	+	+	—	—	—	—	—	—	—	—	—
	6	+	+	—	—	—	—	—	—	—	—	—
	24	++++	++++	+++	++	—	—	—	—	—	—	—
9340	3	+++	+++	+++	++	+	—	—	—	—	—	—
	6	++++	++++	++++	+++	+++	++	+	+	—	—	—
	24	++++	++++	++++	++++	++++	+++	+++	++	++	+	—
9341	3	+++	+++	+++	+++	++	++	—	—	—	—	—
	6	++++	++++	++++	++++	+++	+++	+++	+++	++	—	—
	24	++++	++++	++++	++++	++++	+++	+++	+++	++	—	—

9340 agglutinated the majority strains of of the *B. parapatrificus*. Sixteen out of the eighteen strains of *B. parapatrificus* were markedly agglutinated in the 1:20 dilution, fifteen by the 1:40 dilution, and fourteen by the 1:80 dilution, but as the dilutions increased the number of strains agglutinated decreased rapidly. Three strains approached the titres shown by the two German strains at 1:5120, but two showed only a slight reaction in the lowest dilutions at twenty-four hours.

Table 2 shows that the serum prepare against *B. parapatrificus* 4042Ea likewise agglutinated both strains of "*B. innutritus*" as well as a majority of the strains of *B. parapatrificus*. This was a weaker serum, completely agglutinating the homologous strain

TABLE 2

*Agglutination tests with serum of rabbit 3027 immunized against Bacillus parapatrificus 4042Ea*

CULTURE NUMBER	TIME IN HOURS	DILUTIONS OF SERUM								Con- trol
		20	40	80	160	320	640	1280	2560	
491	3	+	+	-	-	-	-	-	-	-
	6	+	+	-	-	-	-	-	-	-
	24	++++	++++	+++	+++	-	-	-	-	-
1538	3	+	+	-	-	-	-	-	-	-
	6	+	+	+	+	-	-	-	-	-
	24	++++	+++	++	+	-	-	-	-	-
3572Ae	3	-	-	-	-	-	-	-	-	-
	6	+	+	+	-	-	-	-	-	-
	24	++++	++++	+++	+	-	-	-	-	-
3945	3	+	-	-	-	-	-	-	-	-
	6	++	++	+	-	-	-	-	-	-
	24	++++	+++	++	+	-	-	-	-	-
3953	3	+++	++	-	-	-	-	-	-	-
	6	+++	++	-	-	-	-	-	-	-
	24	++++	++++	++	-	-	-	-	-	-
3978B	3	+++	++	+	+	-	-	-	-	-
	6	++++	++++	+++	++	+	-	-	-	-
	24	++++	++++	+++	+++	++	+	-	-	-
4013Cb	3	+	+	-	-	-	-	-	-	-
	6	++	+	+	-	-	-	-	-	-
	24	++++	++++	+++	++	+	-	-	-	-
4042Ea*	3	+++	+++	++	++	+	-	-	-	-
	6	++++	++++	+++	+++	++	+	+	-	-
	24	++++	++++	++++	++++	++++	++	+	+	-
4056Bb	3	+++	++	+	-	-	-	-	-	-
	6	+++	++	++	+	-	-	-	-	-
	24	++++	++++	++++	+++	++	+	-	-	-
4104	3	-	-	-	-	-	-	-	-	-
	6	+	-	-	-	-	-	-	-	-
	24	+++	+++	++	-	-	-	-	-	-

\* Homologous strain of *Bacillus parapatrificus*.

TABLE 2—*Concluded*

CULTURE NUMBER	TIME IN HOURS	DILUTIONS OF SERUM								Control
		20	40	80	160	320	640	1280	2560	
4351	3	—	—	—	—	—	—	—	—	—
	6	+	—	—	—	—	—	—	—	—
	24	++++	+++	+	—	—	—	—	—	—
4388Cb	3	—	—	—	—	—	—	—	—	—
	6	+++	++	++	—	—	—	—	—	—
	24	++++	++++	++++	+++	+	+	—	—	—
4453	3	—	—	—	—	—	—	—	—	—
	6	+	—	—	—	—	—	—	—	—
	24	+++	+++	++	+	+	—	—	—	—
5465	3	—	—	—	—	—	—	—	—	—
	6	+	+	—	—	—	—	—	—	—
	24	++++	+++	+++	+	—	—	—	—	—
8861	3	—	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—	—
	24	+++	+++	++	—	—	—	—	—	—
8862	3	—	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—	—
	24	++++	++++	+++	+++	—	—	—	—	—
8863	3	+++	+++	++	++	+	—	—	—	—
	6	+++	+++	+++	+++	+++	+	+	—	—
	24	++++	++++	++++	++++	+++	+++	+++	+	—
9193	3	—	—	—	—	—	—	—	—	—
	6	++	—	—	—	—	—	—	—	—
	24	++++	+++	+++	++	—	—	—	—	—
9340	3	—	—	—	—	—	—	—	—	—
	6	+	—	—	—	—	—	—	—	—
	24	+++	+++	+	—	—	—	—	—	—
9341	3	—	—	—	—	—	—	—	—	—
	6	+	+	—	—	—	—	—	—	—
	24	+++	+++	+++	+	—	—	—	—	—

only in a dilution of 1:320. Yet all strains were strongly agglutinated at 1:40 and one of "*B. innutritus*" at 1:80.

In each series of tests we observed considerable difference in agglutinability of certain strains. Snyder (1936) noted similar differences and a comparison of his tables and ours shows that certain strains agglutinated weakly with one serum and strongly with another, even though the second serum showed a lower homologous titre.

We are strongly inclined to interpret the variations in agglutinability of certain strains of *B. paraputrificus*, particularly in the three-hour readings, as dependent upon possible differences in flagellar antigens but the whole problem of flagellar and somatic agglutinogens in this species requires further study.

The essential point now is that a serum prepared against a representative strain of "*B. innutritus*" (Kleinschmidt) strongly agglutinated a majority of the available strains of *B. paraputrificus* (Bienstock), while a similar serum prepared against a representative strain of *B. paraputrificus* (Bienstock) strongly agglutinated both available strains of "*B. innutritus*" (Kleinschmidt). In view of their essential identity, morphological, cultural, and serological, we suggest that, on the ground of priority, the name "*Bacillus innutritus*" should be dropped in favor of *Bacillus paraputrificus*.

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# THE EFFECT OF SOFT X-RAY IRRADIATION ON BACTERIOPHAGES

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Although much work has been done on the effects of X-rays on bacteria and other biologic materials, relatively little has been done on bacteriophages. Among the results reported is that of Beckwith, Olson and Rose (1930) who worked with six strains of *Escherichia coli* and seven *Escherichia coli* bacteriophages. They found that exposures to rays from a Roentgen tube for 30 minutes reduced, but did not destroy the lytic activity of 3 of the possible 42 combinations. The X-rays used in their experiments were probably the penetrating or short wave-length radiations of medical radiology so that only a small part of the radiant energy was absorbed by the bacteriophages. If it is the energy absorbed by the bacteriophages which reduces their activity, and if other conditions are the same, one would expect the degree of inactivation to be greater when the X-rays were of the less penetrating long wave-length, or soft, variety. Having at hand a source of such long wave-length X-rays, the authors decided to investigate their effects on several bacteriophages.

## EXPERIMENTAL

Bacteriophages for a hemolytic *Streptococcus*, *Staphylococcus aureus*, *Escherichia coli-communis* and Shiga dysentery bacilli were chosen because of their high lytic activity and ready transmissibility. The radiations were supplied by a gas X-ray tube (Kersten, 1934), having a copper target and window of aluminum foil and cellophane, operated at 30 peak K. V. and 10 m.a. The most intense parts of the radiation transmitted by the window were the  $K_{\alpha}$  (1.54 Å) and the  $K_{\beta}$  (1.38 Å) lines of the copper

X-ray spectrum. Lysis was observed throughout the period of activity by means of a turbidimeter developed by the authors and described in a previous paper (Wright and Kersten, 1937).

The lysates used were prepared by inoculating test tubes, containing 10 cc. of brain-heart infusion broth with 0.5 cc. of 24-hour broth culture of the organisms. This new culture was incubated at 37°C. for 2 hours, 1 cc. of the lysate from a previous run added, and incubation continued until a complete clearing was produced. One cubic centimeter of the young lysate was placed in a small Pyrex cup about 1.5 cm. in diameter. This quantity insured an amount of the lytic principle adequate for producing clearing in normal control tubes within from 2 to 4 hours after having been added to a susceptible culture. The cup, open at the top, was placed directly beneath the window of the X-ray tube so that the surface of the liquid in it was 4.2 cm. from the focal spot. After irradiation, the material was transferred by pipette to a test tube containing a 2-hour broth culture of the homologous bacterium. A similar tube of broth culture to which 1 cc. of non-irradiated bacteriophage was added acted as a lytic control while another culture tube containing no bacteriophage acted as a normal growth control. All tubes were incubated at 37°C. and readings of turbidity were taken every 15 minutes until the maximum clouding or clearing was reached. Figures 1, 2, 3 and 4 show the results of irradiation for 4 different bacteriophages for various periods of time. Each graph shows a normal growth curve in the absence of the lytic agent and other curves for growth in the presence of the lytic agent. During growth the microampere readings increase with increasing clouding, while during lysis, as the culture clears, the microampere readings decrease, until a low reading is obtained upon completion of lysis. Tubes containing bacteriophages irradiated for 1 hour show only slight modification of the lytic activity while those containing bacteriophages which had been subjected to from 4 to 6 hours of irradiation have growth curves approximating those of their respective normal ones. Those irradiated for periods between these extremes have intermediate growth curves.

With the X-ray tube used, the irradiated sample is warmed slightly by the heat which passes through the window with the X-rays. In order to show that the effect of irradiation on the lytic activity of the bacteriophages was due to the X-rays and not to the small quantity of heat accompanying them a number of additional experiments were conducted. The temperature just beneath the window of the X-ray tube was measured by means of a thermocouple and was found to be less than 37°C. during a 5-hour period of irradiation. In another experiment the bacteriophage was held at a temperature below 14°C., by means of an ice-salt mixture, during irradiation. Reduction of the lytic

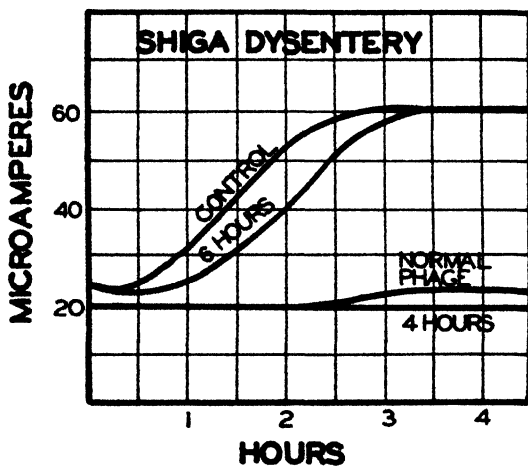


FIG. 1

activity was of the same degree as that of an uncooled bacteriophage irradiated for the same length of time. A similar bacteriophage cooled during the irradiation period in an identical manner, but protected from X-rays showed no alteration in its ability to produce lysis. Bacteriophages were also incubated for 5 hours at 45°C. and found still to possess their normal lytic activity. From these experiments it may be concluded that the bacteriophages were not inactivated by heat from the X-ray tube.

There was also the possibility of an inhibiting substance being produced in the broth by the long periods of irradiation (Blank and Kersten, 1935). To investigate this possibility, 1 cc. of



double-strength broth was irradiated for 4 hours, 1 cc. of bacteriophage added, and the mixture held at 37°C. for 4 hours. This mixture was then added to a susceptible young culture. No

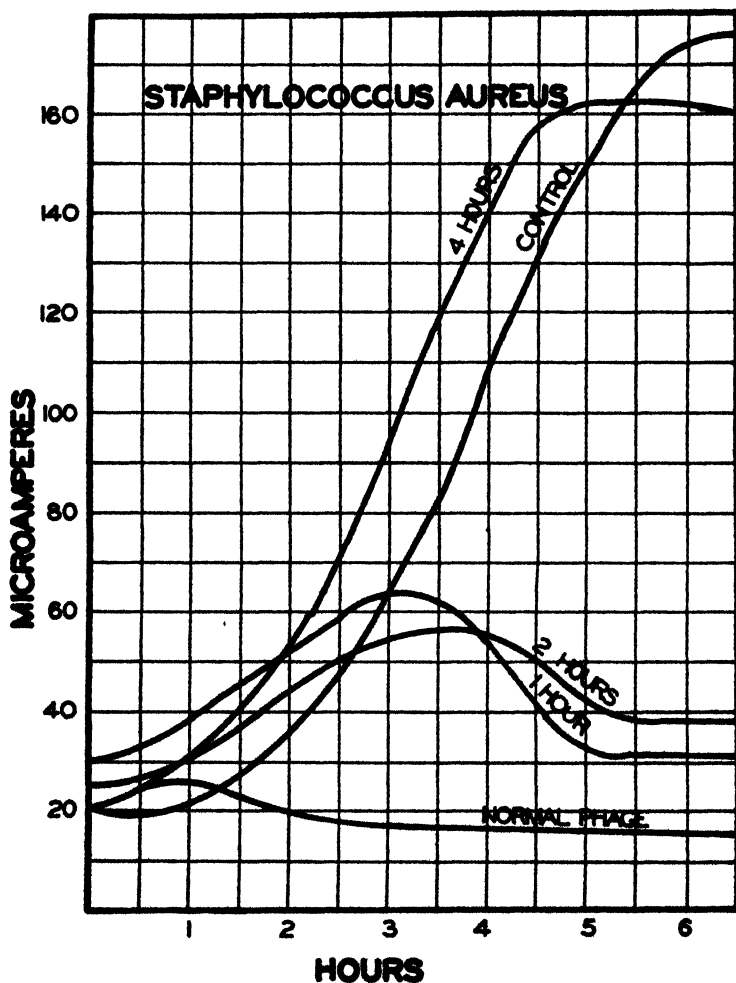


FIG. 2

reduction of lytic activity was produced, indicating the absence of inhibiting substances in the irradiated broth.

Finally there was the possibility of the pH of the medium being changed by irradiation and this in turn affecting the activ-

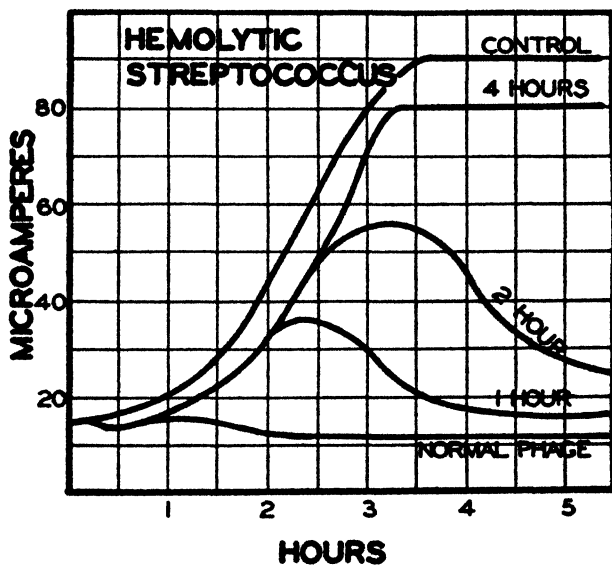


FIG. 3

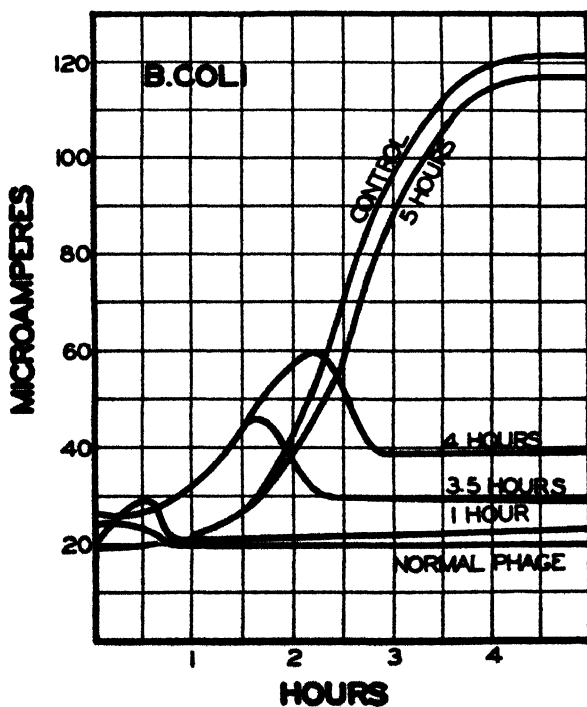


FIG. 4

ity of the bacteriophages. Broth which had been irradiated for 4 hours showed no change in pH value as compared with non-irradiated broth from the same tube, when matched with standard brom-thymol-blue indicator ampoules.

It is interesting to note that the growth curves obtained using diluted bacteriophages were found to be similar in shape to those obtained using bacteriophages which had been partially inactivated by irradiation with soft X-rays, and the bacteriophages which were active in high dilutions required the larger doses to produce inactivation.

#### CONCLUSIONS

From these results it may be deduced that: (1) the lytic activity of bacteriophages for *Staphylococcus aureus*, hemolytic *Streptococcus*, *Escherichia coli* and Shiga dysentery may be partially or completely inhibited by exposure to soft X-rays; (2) that all of these bacteriophages are not equally affected by the same dose of X-rays; (3) that the degree of inhibition for any one of these bacteriophages increases with increased doses of X-rays.

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# A DIFFERENTIAL MEDIUM FOR THE MENINGOCOCCUS AND GONOCOCCUS<sup>1</sup>

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The meningococcus and gonococcus often give rise to lesions in the human being which are of the gravest import. It is, therefore, highly desirable that a differential diagnosis between these two and other gram-negative cocci should be made as early as possible. Workers in hospital laboratories have encountered various difficulties in the practical differentiation of these organisms. The difficulties lie mainly in the slowness of acid production on solid media in general use, and in the frequent failure of the organisms to multiply in fluid media. Numerous attempts have already been made to remedy these difficulties.

After a considerable study of the factors influencing the growth and growth products of *Neisseria*, a medium has been obtained which we believe offers a practical means by which an early differential diagnosis can be made.

The medium to be described consists of whole serum from which the fermentable substances and diastase have been removed and to which have been added a concentrated broth containing inorganic salts and a carbohydrate and indicator. Differential identification can usually be established in 12 to 24 hours.

## REVIEW OF LITERATURE

Bumm (1885), who first successfully cultivated the gonococcus, grew it on solidified human placental blood. Later investigators added unheated proteins to agar for the cultivation of

<sup>1</sup> Submitted to the Graduate School of the University of Pittsburgh in partial fulfillment of the requirements for the degree of Master of Science.

both the gonococcus and meningococcus. Dunn and Gordon (1905), Rundle, Mottram and Williams (1907), Symmers and Wilson (1907), Shennan and Ritchie (1908), Elser and Huntoon (1909), Swartz (1920), and Torrey and Buckell (1922) used hydrocele and ovarian fluids. Martin (1911), and Jenkins (1921) used human serum. Kinsella, Broun and Garcia (1923) used beef serum. Jenkins (1924), MacNaughton (1923), and Herrold (1928) utilized whole blood. North (1909) reported good results with gelatin-agar, and Vedder (1915) with corn-starch agar. Watabiki (1916) added whey to agar. Cole and Onslow (1916), on the other hand, reported best results with milk casein mixed with extract of pig pancreas.

In 1922 Erickson and Albert reported the use of beef testicular blood-agar and in 1926 Pelouze and Viteri obtained excellent results with brain broth medium without addition of blood. Cole and Lloyd (1916-17) used "tryptamine" blood extract in agar and in broth. They were particularly interested in the amino-acid content of the medium as were also McLeod, Wheatley and Phelon (1927).

Although the type of protein utilized has been stressed by most investigators, Martin (1911), and Phelon, Duthie and McLeod (1927) indicated the importance of reaction of the medium. The latter authors called attention to the ill effects of unduly alkaline media on both the meningococcus and gonococcus. Wherry and Oliver (1916), and Chapin (1918) believed that an altered oxygen tension was also a major factor in the cultivation of gonococci.

Perhaps the media most extensively used for differentiation of gonococcus and meningococcus are the following: the serum water medium of Hiss, the semisolid agar of Torrey and Buckell (1922), the ascitic fluid of Elser and Huntoon (1909), Gradwohl (1926), and others, the sugar-free infusion-broth agar of Martin (1911), the hormone agar of Huntoon (1918), and the brain agar of Pelouze and Viteri (1926). These various media, with and without modifications, have been used in our laboratories for several years, but since growth requirements do not entirely parallel those of acid production they have all proved more or

less unsatisfactory in our hands, chiefly because of irregular or tardy reactions. Likewise, the egg-white mediums of Le Soudier and Verge (1925) and of Roublot (1925) were tested as a basis for a differential medium but they could not be used because of interference with the action of the indicator.

It was evident, therefore, that none of the methods which we have tried were entirely satisfactory for diagnostic purposes, especially in the hands of the student in bacteriology.

Some of our preliminary observations indicated that coagulated serum might possess the requisite qualities. These observations were extended in various ways and resulted in the development of the medium described in the following pages.

#### PREPARATION OF MEDIA

In these experiments, sera from beef, calf, lamb and hog were tested. In general, media prepared from each of the sera yielded equally satisfactory results. Beef serum being more easily obtainable was, therefore, more extensively used. Blood for this purpose was collected in the usual manner and the serum separated from the clot.

A mixture of three parts of serum and one part of sterile water (250 to 300 cc. in a 500-cc. Florence flask) was heated in a water bath at 65°C. for one hour, with frequent shakings, on two consecutive days. Enough water was kept in the bath to float the flask in order to prevent coagulation at the bottom. The mixture was cooled to 40°C. or below and inoculated heavily with a 24-hour culture of *Escherichia coli-communis*. It was then incubated at 37°C. for four or five days or until a secondary alkalinity of pH 7.7 to 7.9 had occurred. The mixture was again heated in the water bath at 65°C. for one hour to kill the organisms present. At this point the sterilized mixture could be stored in the icebox until it was convenient to complete its preparation. The serum water mixture thus treated was found to be deficient in growth-promoting factors but was very satisfactorily used as a basic medium to which various substances were added in an attempt to secure a medium which would produce luxuriant growth and a good differentiation, and would prolong the life

of the organisms. Various materials were added to the basic medium, singly and in combinations, and the effects upon the cultures of meningococci and gonococci were studied. The chemicals studied most extensively were: potassium sulphate, magnesium sulphate, sodium sulphate, sodium sulphite, potassium chloride, calcium chloride, magnesium chloride, sodium nitrate, sodium bicarbonate, monobasic and dibasic potassium phosphate and sodium phosphate.

Growth and acid production of both meningococci and gonococci were slightly accelerated by additions of sodium sulphate, potassium sulphate, potassium chloride, sodium nitrate and sodium bicarbonate in amounts varying from 0.1 to 0.3 per cent. Of the combinations of salts studied, those which affected acid production most were mixtures of sodium or potassium sulphate with calcium chloride and sodium bicarbonate or sodium nitrate. In some instances we were able to detect acid production in media containing salts 4 to 12 hours earlier than in those containing no salts. Further additions of meat extract, peptone or neopeptone were tried. These latter substances so enhanced the growth of both organisms that a mixture of meat extract with one of the peptones has been employed, neopeptone being preferred.

Several indicators were tested, the most important being: azolitmin, Andrade's, phenol-red, brom-thymol-blue, brom-cresol-purple, and cresol-red. Satisfactory results were obtained with the last five but only when culture tubes were sealed with corks or rubber stoppers.

When the Andrade's indicator was employed, it was necessary to add 1 cc. to 100 cc. of medium. The phenol-red indicator required 5 cc. of 0.04 per cent aqueous solution to each 100 cc. of medium. Cresol-red, brom-cresol-purple and brom-thymol-blue each gave optimum results by the addition of 2.5 cc. of 0.1 per cent aqueous solution to each 100 cc. of medium.

Brom-thymol-blue gave somewhat more satisfactory readings than the others and was, therefore, the indicator of choice.

In order to incorporate into the medium the most desirable ingredients studied, it was found necessary to add to the serum-

water mixture a special concentrated broth which was prepared as follows:

Water...	1000 cc.
Neopeptone or peptone.....	50 grams
Meat extract.....	10 grams
Sodium chloride. . . . .	10 grams
Sodium sulphate .....	10 grams
Sodium nitrate .. . . .	5 grams

The mixture was heated until dissolved, reaction unadjusted, and then sterilized at 10 pounds pressure for 15 minutes.

The formula for the completed medium consisted of:

Serum-water mixture (as prepared above).....	800 cc.
Concentrated broth (as prepared above) . . . . .	200 cc.

The reaction was adjusted to pH 7.8.

Carbohydrate (glucose, maltose, etc.)... . . . .	10 grams
Brom-thymol-blue 0.1 per cent aqueous solution... . . . .	25 cc.

Sodium hydroxide was used for adjusting the reaction when the medium was too acid. Additional peptone was added when the medium was too alkaline. The medium was tubed in 5-cc. amounts and coagulated in the inspissator. Inspissation of the medium was best accomplished by having the initial temperature of the water in the inspissator jacket between 70° and 75°C. and raising this to 90°C. in 45 minutes to one hour. This temperature was maintained until coagulation was completed. The time required to complete coagulation depended upon the number of tubes in the inspissator.

As soon as tubes were cooled enough to permit handling they were removed from the inspissator, placed in an autoclave in slanted position and heated for 30 minutes at two pounds pressure. They were then removed from the autoclave, incubated overnight at 37°C. and re-heated for 30 minutes at two pounds pressure. This was sufficient to sterilize the medium. The tubes were then stoppered tightly with sterile corks or rubber stoppers and stored at room temperature in the dark, since light slowly decolorizes the indicator.



A medium prepared and stored in this manner for longer than one year continues to give excellent results.

#### GENERAL RESULTS

The fermentation reactions of 56 strains of gonococci and 51 strains of meningococci have been repeatedly studied on this medium. Thirty-nine strains of the former and 41 strains of the latter were recently isolated from hospital cases. The remaining strains were from stock cultures. The carbohydrates found to be of most value in the differentiation of meningococci and gonococci were those commonly used with other methods referred to, namely, glucose and maltose in 1 per cent concentration. As the work progressed, other carbohydrates were tested but these added nothing of practical value to the information obtained by the use of the two mentioned. In cultures of meningococci which were tightly stoppered, fermentation occurred in glucose and maltose in from 4 to 16 hours with a definite change in color of the indicator.

Sealed cultures of gonococci fermented glucose in 8 to 24 hours. No further change occurred in cultures of either organism during the first four days, but on the fifth day both gonococci and meningococci produced acid in mannitol in all strains studied. This reaction was probably due to impurities in the carbohydrates. No fermentation was obtained in sucrose, lactose, salicin, sorbitol, dulcitol, mannose, levulose and galactose by either organism. Prolonged incubation produced little or no color change in uninoculated controls and in those cultures with non-fermented sugars.

Other investigators have reported the increased amount of growth produced by the gonococcus when tubes are sealed prior to incubation. We noted, however, that when shallow stabs were made into the medium rather than surface inoculation alone, acid appeared almost as early as when the tubes were sealed.

Sealing the tubes did not favorably influence the growth of meningococci, but when cotton plugs were employed all cultures of this organism produced a secondary alkalinity in fermented

sugars within 48 hours. Three strains of gonococci which grew very luxuriantly produced a slight secondary alkalinity in glucose medium. With the remaining 53 strains studied, the acidity was maintained over a period of weeks. Two factors are concerned with production of these phenomena. These are oxygen tension and the prevention of escape of any gas or volatile acid. Qualitative analysis, similar to that employed by Phelon, Duthie and McLeod (1927), except that barium hydroxide was substituted for soda lime in the last two tubes, gave a positive test for carbon dioxide. Negative results were obtained in the tests for volatile acids. Loss of the carbon dioxide probably explains the secondary alkaline reactions, although the possibility of ammonia production was not ruled out.

Six strains of *Micrococcus catarrhalis*, three strains of *Micrococcus pharyngissicus* and eight strains of chromogenic gram-negative diplococci were studied for comparison with the meningococci and gonococci strains. No carbohydrates were fermented by *Micrococcus catarrhalis* in tubes plugged with cotton, but when culture tubes were sealed, even those controls to which no carbohydrate and no salts were added showed an acid reaction. This latter phenomenon was present with all strains of *Neisseria* studied except meningococci and gonococci. Therefore, these mediums can be used to differentiate only the latter two organisms from each other and from others of this group.

#### DISCUSSION

For the differentiation of microorganisms, morphological, cultural, serological and fermentative methods are commonly employed. At present no one of these alone is sufficiently comprehensive. Fermentation, together with morphology, is probably most frequently used in routine diagnosis. Fermentation still presents many difficulties in the *Neisseria*. These bacteria have become so highly differentiated that the physical and chemical properties of the medium must be carefully adjusted in order to permit optimal conditions for growth and ready utilization of carbohydrates for acid production.

The two organisms under study have such a high degree of

specialization that they are capable of utilizing only certain proteins. In contradistinction to all other bacteria studied, including several members of the streptococcic, staphylococcic, diphtheria and colon-typhoid groups, the meningococci and gonococci, in sealed cultures, produced no change on the coagulated blood serum which had been depleted of fermentable substances and to which had been added an indicator but no carbohydrate. As has already been noted, other members of the *Neisseria* produced acid reaction in this medium. Based on these data, it would appear that most other forms of bacteria are able to hydrolyze complex proteins into simpler compounds such as proteose, polypeptide, peptide, etc., and from among the simpler organic compounds certain fractions are utilized and others are eliminated as dissimilation products comparable to the findings of Kluyver (1931). The presence of these dissimilation products may explain acid production in control tubes. On the other hand, meningococci and gonococci do not have the property of hydrolyzing complex proteins in order to secure the selective substances for growth. These two organisms can utilize only such simple protein substances as are directly available for assimilation.

The question of amino-acids has received consideration by various investigators in connection with this group of bacteria. The value of Cole and Onslow's medium (1916) for the cultivation of the gonococcus was largely attributed by Cole and Lloyd (1916-17) to its abundance of amino-acids. The latter investigators stated, "The value of the addition of peptone to a nutrient medium is almost entirely due to the fact that all commercial samples contain small amounts of amino-acids in the free state which are directly available for assimilation." The fact that dialyzed trypsin digest failed to support growth of the gonococcus was attributed by Miller, Hastings and Castles (1932) to the removal of amino-acids.

McLeod, Wheatley and Phelon concluded that among the elements essential to the growth of the gonococcus were, "Some sources of amino nitrogen and salts such as are present in meat extract." They further stated, however, that large amounts of amino-acid inhibit growth.

Whatever these growth-promoting substances may be, they are present in peptone, neopeptone and meat extract.

It is debatable whether such substances are amino-acids or nitrogen compounds of simpler constitution. That such simple compounds, as nitrates, may be directly usable for growth is indicated by the fact that among the various inorganic salts tested the greatest acceleration of growth was obtained by addition of sodium nitrate to the culture medium. These findings agree in part with those reported by Miller, Hastings and Castles (1932). They found bicarbonate to be essential to the growth of gonococci. No detailed study was made to ascertain whether this was due to the kation or anion or what part was played by valency. At present it is not clear how this result is brought about. Obviously osmotic pressure is increased and this may act in a greater utilization of available food material. A more plausible explanation is the specific effect of certain ions on enzymes associated with growth and acid production. Numerous instances of the enhancing effect of ions in biochemical activity might be cited. Waldschmidt-Leitz (1929) and Haldane in their studies on enzymic action in the animal and in the plant organism illustrated the effect of various inorganic salts on specific enzyme production. Glick and King (1932) showed the inhibiting effect of certain organic compounds on liver esterase. Karström (Stephenson, 1932) has established the fact that bacterial species are not constant in regard to their enzymic content, but vary sharply according to their nutritional environment, and that certain of the sugar-splitting enzymes are present in demonstrable amounts only if the organism has been grown in the presence of its specific substrate.

### *Oxygen tension*

The two main factors involved in the increased acid production in sealed cultures are lowered oxygen tension and loss of any volatile substances. Experiments indicated that retention of carbon dioxide is probably the essential differential factor concerned in the meningococcus cultures. The rôle played by each of these two factors in gonococcic cultures is not so easily determined. Wherry and Oliver (1916) and Chapin (1918) claimed

reduced oxygen tension to be an important growth promoting factor in the gonococcus. On the other hand, Torrey and Buckell (1922), and Erickson and Albert (1922) believed that a reduced oxygen tension was not necessary. They attributed the increased growths obtained in partially anaerobic cultures to the changed physical conditions brought about by these methods, the retention of moisture being the condition most stressed. The observations made in this study support the contention that an altered oxygen tension, such as can be produced by methods described, does enhance growth as well as prevent escape of moisture and volatile substances. In unsealed cultures, growth along the edge of the water of condensation was no more luxuriant than at any other place on the slant. As has already been noted, shallow stabs made at any place on the surface resulted in an early production of acid, indicating the presence of growth.

#### SUMMARY

The preparation of a differential medium for the diagnosis of meningococcus and gonococcus has been described. Essential features of this medium are a combination of serum from which fermentable substances and diastases have been removed and to which have been added a concentrated broth containing certain inorganic salts. Having the culture tubes sealed during growth was found to be necessary in order to insure the continued presence of acid in the medium.

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# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## NEW YORK CITY BRANCH

TENTH MEETING, NEW YORK UNIVERSITY MEDICAL SCHOOL,  
OCTOBER 19, 1937

### EFFECT OF BACTERIAL FILTRATES IN THE PROPHYLAXIS OF UPPER RESPIRATORY INFECTIONS. A. S. Gordon.

Bacterial filtrates, or soluble toxins, yielded by the microorganisms commonly found in the upper respiratory tract, were used in an attempt to prevent, or reduce the incidence of, upper respiratory infections, particularly the common cold. The individual's susceptibility is considered the most important single factor in the production of the disease. The author believes that the bacterial filtrates or metabolites of these bacteria are more valuable than the bacterial vaccines, and that these soluble proteins possess more potent antigenic properties than the suspensions made from the bacterial bodies.

The method used was as follows: Broth cultures were made from the nasal passages, sinus punctures, crypts of tonsils, and the nasopharynx. The metabolites were separated from the bacteria by filtration, and were modified with formalin. Patients were tested intradermally to the filtrates, and the degree of reaction was used as a guide in the dosage and frequency of inoculation. The injections were given in the early Fall season. A group of highly susceptible patients were kept under observation and treat-

ment during the past four years and the inoculations were repeated every autumn. The number of injections ranged from three to twelve and were administered once or twice a week, the amount varying from 0.2 to 0.5 cc. per injection of the solution.

The results are highly satisfactory and the study is being continued.

### THE INCIDENCE OF *MONILIA ALBICANS* IN ROUTINE SPUTUM SPECIMENS. Frederick R. Weedon, Dorothy Kenney and Marie E. Shirk, Bureau of Laboratories, Yonkers, New York.

In view of the accepted association of *Monilia albicans* with inflammatory lesions in various sites, it seems worthwhile to collect data upon the occurrence of this yeast in specimens of various sorts submitted for examination for any reason. The present series is a study of sputum from fifty-five cases each of which presented symptoms of chronic pulmonary involvement of some degree, the specimens being submitted for examination for the presence of tubercle bacilli.

Of these fifty-five specimens five contained *Monilia albicans*. Other yeasts and non-pathogenic fungi were found frequently and a survey of these is being undertaken.

If this high incidence of *Monilia albicans* persists in a series of adequate



length, it is hoped that an attempt to correlate the clinical findings can be made later in order to determine the significance of the phenomenon.

ACTIVE HALOGEN COMPOUNDS OF THE WAR GAS TYPE AS FUNGICIDES AND BACTERICIDES. THE LACRIMATOR MONOIDOACETONE. *Frederick R. Weedon*, Bureau of Laboratories and Yonkers Professional Hospital, Yonkers, New York.

Since mildly active halogen compounds are well established as fungicides and bactericides and since practically all successful war gases are simply very reactive halogen compounds it seemed likely that these latter might have similar effect against microorganisms.

In the halogen series toxicity increases from fluorine through chlorine and bromine to iodine. This is well exemplified by three of the compounds studied, chloro-, bromo- and iodoacetophenone which, as 1 per cent solutions in alcohol, were tested against *Penicillium* spores in wort agar plates. A strip of filter paper 3 x 0.8 centimeters was moistened with each and placed on the seeded agar near the periphery. On the plate with the chlorine compound 500 colonies grew, that with the bromine compound supported 25 while on that with the iodine compound there were no colonies.

Problems of toxicity, stability, vola-

tility, solubility and lacrimatory effect have been met by the study of such factors as varying solvents, temperature, light, pH, etc., and by animal experiment. Iodoacetone has been most extensively studied. An exposure of *Penicillium* spores or of *Staphylococcus aureus* 209 to a concentration of one to five hundred thousand iodoacetone in acetone for five minutes kills these organisms. An ointment using one part per thousand of this substance is yellow petrolatum with about 10 per cent acetone and a varying amount of lanolin has been in routine use for nearly four years in one of the local clinics for skin diseases and has been satisfactory in healing fungous infections of the skin including those in the scalp caused by *Microsporon falinum*. Very interesting results have been obtained in scattered cases of other, more serious fungous diseases which will be reported.

Ethyl iodoacetate in ethyl acetate is an excellent fungicide in practical use as a one to one thousand ointment as well as in the test tube.

Iodonitromethane is a good fungicide so far but little studied. Perhaps the most promising compound so far studied is iodoacetophenone or phenacyl iodide. Therapeutically it seems at least as effective as iodoacetone and certain physical and chemical considerations may act to make it more so.

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